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INSTITUTE
FOR
CANCER
RESEARCH

**ASPP1 AND ASPP2 LINK THE RAS AND p53
SIGNALLING PATHWAYS**

by

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A thesis submitted in fulfilment
of the requirements for the degree of

Doctor of Philosophy

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Abstract

In this thesis, the regulation of ASPP1 and ASPP2 was investigated. ASPP1 and ASPP2 are p53 co-activators that can specifically induce p53-dependent apoptosis but have no effect on p53-dependent cell cycle arrest. Both ASPP1 and ASPP2 contain a Ras-association domain in their amino terminal regions. ASPP1 can bind activated Ras directly via its amino terminal region *in vitro*, and both endogenous ASPP proteins bind endogenous Ras *in vivo* after stimulation of cells with serum and growth factors. Oncogenic H-RasV12 and K-RasV12 stimulate ASPP1 and ASPP2 pro-apoptotic activity in a p53-dependent manner and can also stimulate ASPP2 co-activation of the p53 family members, p63 and p73. These results suggest that ASPP1 and ASPP2 are novel Ras effector proteins.

Ras is upstream of several effector pathways. One of its downstream effector pathways, Raf-MEK-MAPK, can activate ASPP1 and ASPP2. MAPK phosphorylates ASPP2 *in vitro*, and both ASPP1 and ASPP2 *in vivo* at serines 746 and 827, respectively. ASPP1 and ASPP2 phosphorylation by MAPK results in an increase in their ability to co-activate p53. Additionally, MAPK phosphorylation of ASPP2 leads to increased ASPP2 protein levels, suggesting that MAPK can regulate ASPP2 by modulating its protein stability.

ASPP1 and ASPP2 deletion fragments were used to examine the regulation of ASPP proteins. Amino-terminus fragments were shown to increase full-length ASPP activity when co-transfected. Moreover, PKA was also found to be a regulator of ASPP2 and was shown to phosphorylate ASPP2 *in vitro*. Forskolin, a stimulator of PKA, could enhance ASPP2 activity. The results provide the first insight into these novel mechanisms by which ASPP activity may be regulated.

To my parents and to Terry
For their constant love and support

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Declaration

All the work presented in this thesis is the result of my own work unless otherwise stated and does not constitute part of any other thesis. The work herein described was carried out while I was a graduate student at the Ludwig Institute for Cancer Research, Imperial College School of Medicine at St Mary's Hospital, under the supervision of Prof. Xin Lu and Prof. Martin Allday.

Nadia Godin-Heymann

Table of Contents

ABSTRACT 2

ACKNOWLEDGEMENTS 4

DECLARATION..... 5

TABLE OF CONTENTS 6

FIGURES AND TABLES 11

LIST OF ABBREVIATIONS 15

CHAPTER 1: INTRODUCTION 19

1.1 Cancer 19

1.2 Cell cycle 23

1.2.1. Rb regulation of the cell cycle 29

1.2.2. p53 regulation of the cell cycle 34

1.3 Apoptosis 36

1.3.1. Caspases..... 38

1.3.2. Regulation of caspases through the death receptor pathway..... 39

1.3.3. Regulation of caspases through the mitochondrial pathway 42

1.3.4. Inhibitors of apoptosis proteins (IAP)..... 45

1.4. p53 46

1.4.1. p53: structure-function relationship 47

1.4.2. p53 as a transcription activator 54

1.4.3. p53 as a transcription repressor 62

1.4.4. p53 can induce apoptosis in a transcriptionally-independent manner..... 64

1.4.5. Regulation of p53 65

1.4.6. Choice of response: cell cycle arrest or apoptosis 81

1.4.7. p53 and tumourigenesis 85

1.5. The p53 family members: p63 and p73 89

1.5.1. Are p63 and p73 tumour suppressor proteins? 91

1.5.2. Regulation of p63 and p73..... 92

1.5.3. Crosstalk between p53-family members 94

1.6. The ASPP family.....	96
1.6.1. ASPP1 and ASPP2.....	96
1.6.2. iASPP	103
1.7. Ras	105
1.7.1. Ras: structure-function relationship	105
1.7.2. Regulation of Ras	108
1.7.3. The Raf-MAPK pathway.....	113
1.7.4. PI3K pathway.....	119
1.7.5. Ral GDS pathway.....	122
1.7.6. Other Ras effector pathways	122
1.7.7. Cross-talk between Ras effectors	126
1.7.8. Ras and tumourigenesis.....	127
1.7.9. Ras regulation of cell cycle	131
1.7.10. Choice of response: to die or not to die?	134
1.8. Ras & p53	139
1.9 Aim of study	145
 CHAPTER 2: MATERIALS & METHODS.....	 146
2.1 Material	146
2.1.1. Reagents	146
2.1.2 SDS-PAGE gels.....	160
2.1.3. Antibodies.....	161
2.1.4. Plasmids	163
2.1.5. Cell lines.....	166
2.2. Methods	169
2.2.1. Tissue Culture	169
2.2.1. DNA Techniques	170
2.2.3. RNA Manipulations.....	177
2.2.4. Protein Manipulations	179
2.2.5. Cell-based assays.....	184
2.2.7. In vitro assays	187
2.2.8. Data Analysis	191

CHAPTER 3: ACTIVATED RAS BINDS TO AND STIMULATES THE ACTIVITY OF ASPP1 AND ASPP2.....	192
3.1 Introduction	192
3.2 Results	195
3.2.1. <i>The amino terminus region of ASPP2 is necessary for its full activity.....</i>	195
3.2.2. <i>The amino terminal regions of ASPP1 and ASPP2 contain putative Ras-association domains</i>	197
3.2.3. <i>Ras mutation status in H1299, U2OS and Saos2 cells</i>	199
3.2.4. <i>The amino terminus of ASPP1 binds directly to Ras-GTP preferentially to Ras-GDP</i>	201
3.2.5. <i>Endogenous Ras binds the amino terminus of ASPP1 in vivo</i>	205
3.2.6. <i>Endogenous Ras stimulated with EGF binds ASPP1 and ASPP2</i>	207
3.2.7. <i>Oncogenic H-RasV12 and K-RasV12 stimulate ASPP1 and ASPP2 activity.....</i>	211
3.2.8. <i>Oncogenic Ras does not confer increased reporter specificity to ASPP2</i>	217
3.2.9. <i>ASPP2 stimulation of all p53 family members is increased in the presence of H-RasV12.....</i>	219
3.2.10. <i>ASPP2 co-localizes with H-RasV12 and K-RasV12</i>	221
3.2.11. <i>K-RasV12 changes the sub-cellular localization of ASPP1</i>	223
3.2.12. <i>Oncogenic Ras can transactivate pro-apoptotic genes via endogenous p53 and ASPP.....</i>	225
3.2.13. <i>Endogenous Ras is necessary for full ASPP1 and ASPP2 activity</i>	228
3.3 Discussion	241
3.3.1. <i>ASPP1 and ASPP2 bind activated Ras in vitro and in vivo</i>	241
3.3.2. <i>Oncogenic H-RasV12 and K-RasV12 stimulate ASPP1 and ASPP2 activity.....</i>	242
3.3.3. <i>Oncogenic Ras increases ASPP2 stimulation of p53 family members</i>	242
3.3.4. <i>Endogenous Ras is necessary for full ASPP1 and ASPP2 activity</i>	243
3.3.5. <i>Cell staining shows a difference in ASPP1 and ASPP2 response to oncogenic Ras</i>	246
3.4. Scope of study	249

CHAPTER 4: RAS STIMULATES ASPP1 AND ASPP2 VIA THE RAF-MEK-MAPK PATHWAY	250
4.1 Introduction	250
4.2 Results	254
4.2.1. <i>Ras activates ASPP2 via the Raf pathway</i>	254
4.2.2. <i>ASPP1 and ASPP2 have putative MAPK phosphorylation sites.....</i>	256
4.2.3. <i>ASPP2 is phosphorylated in vitro by MAPK</i>	258
4.2.5. <i>Endogenous MAPK activity is necessary for full ASPP activity.....</i>	265
4.2.6. <i>K-RasV12-dependent change of ASPP1 sub-cellular localization is mediated by MAPK activity</i>	277
4.2.7. <i>Endogenous ASPP1 does not change localization after EGF stimulation</i>	279
4.2.8. <i>ASPP2 is stabilized by the Ras-Raf-MAPK pathway via phosphorylation at its serine 827</i>	281
4.2.9. <i>ASPP2 is not necessary for MAPK activation in MEFs.....</i>	288
4.2.10. <i>Doxorubicin induces apoptosis partially via the MAPK pathway</i>	290
4.2.11. <i>ASPP2 (S827A) co-localizes with K-RasV12.....</i>	292
4.3 Discussion	294
4.3.1. <i>Ras stimulates ASPP2 via the Raf pathway</i>	294
4.3.2. <i>ASPP2 is phosphorylated by MAPK in vitro and in vivo</i>	294
4.3.3. <i>MAPK activity is necessary for full ASPP1 and ASPP2 activity</i>	296
4.3.4. <i>MAPK phosphorylation of ASPP2 increases its stability.....</i>	299
4.3.5. <i>ASPP2 is not necessary for MAPK stimulation in MEFs.....</i>	303
4.3.6. <i>Endogenous MAPK activity is necessary for full apoptotic response to doxorubicin</i>	304
4.3.7. <i>ASPP2 (S827A) co-localizes with K-RasV12.....</i>	305
4.4. Scope of study.....	306

CHAPTER 5: REGULATION OF ASPP1 AND ASPP2 ACTIVITY	307
5.1 Introduction	307
5.2. Results.....	310
<i>5.2.1. The amino terminus of ASPP2 stimulates p53 activity.....</i>	<i>310</i>
<i>5.2.2. The amino terminus of ASPP1 or ASPP2 stimulates p53 activity via full-length ASPP1 and ASPP2</i>	<i>313</i>
<i>5.2.3. Only the amino terminus fragments of human and mouse ASPP can stimulate p53 and full-length ASPP2 activity.</i>	<i>315</i>
<i>5.2.4. ASPP2 (1-360) stimulates ASPP2 wild type and ASPP2 (S827A) to the same extent.</i>	<i>317</i>
<i>5.2.5. ASPP2 is the putative substrate of several kinases</i>	<i>319</i>
<i>5.2.6. ASPP2 is phosphorylated in vitro by p90rsk, PKA and p38 SAPK</i>	<i>321</i>
<i>5.2.7. PKA phosphorylates ASPP2 in vitro on serine 737.....</i>	<i>323</i>
<i>5.2.8. ASPP2 (S736,737A) phosphorylation mutant is as active as ASPP2 wild type</i>	<i>327</i>
<i>5.2.9. Forkolin stimulates both ASPP2 wild type and ASPP2 (S736,737A) activity</i>	<i>332</i>
5.3. Discussion	335
<i>5.3.1. The amino termini of ASPP1 and ASPP2 stimulate full-length ASPP1 and ASPP2 activity</i>	<i>335</i>
<i>5.3.2. ASPP2 is a putative substrate to PKA kinase</i>	<i>338</i>
 CHAPTER 6: FINAL SUMMARY AND DISCUSSION.....	 340
6.1. ASPP1 and ASPP2 are novel Ras effectors.....	340
6.2. ASPP1 and ASPP2 are novel MAPK substrates	342
6.3. Linking the two pathways – is there a feedback loop?	344
6.4. A novel pathway for Ras-mediated apoptosis.....	347
6.4. Therapy.....	353
6.5. Summary of achievements in this study	354
 REFERENCES	 355

Figures and Tables

Chapter 1: Introduction

Figure 1.1. <i>Cyclins and the cell cycle</i>	25
Figure 1.2. <i>Caspase activation via multiple pathways</i>	41
Figure 1.3. <i>p53 structure</i>	50
Figure 1.4. <i>p53 target genes</i>	58
Figure 1.5. <i>Post-translational modifications of human p53</i>	70
Figure 1.6. <i>ASPP1 and ASPP2 structure and function</i>	98
Figure 1.7. <i>The Ras-Raf-MEK-MAPK pathway</i>	117
Figure 1.8. <i>The Ras-PI3K signalling pathway</i>	121
Figure 1.9. <i>Ras downstream effector pathways</i>	125
Figure 1.10. <i>Ras and p53 crosstalk</i>	141

Chapter 3: Activated Ras binds to and stimulates the activity of ASPP1 and ASPP2

Figure 3.1. <i>The amino-terminal 130 amino acids of ASPP2 is crucial for its full activity</i>	196
Figure 3.2. <i>ASPP1 and ASPP2 contain a putative Ras-association domain in their amino-termini</i>	198
Figure 3.3. <i>Ras mutation status in H1299, Saos2 and U2OS</i>	200
Figure 3.4. <i>Purified amino terminus of ASPP1 binds directly to Ras-GTP with a higher affinity than to Ras-GDP</i>	203
Figure 3.5. <i>Endogenous Ras binds the amino-terminus of ASPP1 in vivo</i>	206
Figure 3.6. <i>Endogenous Ras binds induced ASPP2 after EGF stimulation</i>	208
Figure 3.7. <i>Endogenous Ras binds endogenous ASPP1 and ASPP2 after EGF stimulation</i>	210
Figure 3.8. <i>H-RasV12 and K-RasV12 stimulate ASPP2 activity to the same extent</i>	212
Figure 3.9. <i>Oncogenic H-RasV12 increases ASPP2 activity whereas dominant negative H-RasN17 inhibits ASPP2 activity</i>	214
Figure 3.10. <i>ASPP1 and ASPP2 are activated to a similar extent by H-RasV12 and K-RasV12</i>	216

Figure 3.11. <i>H-RrasN17 and H-RasV12 do not confer additional reporter specificity to ASPP2</i>	218
Figure 3.12. <i>Oncogenic H-RasV12 increases ASPP2 stimulation of all p53 family members</i>	220
Figure 3.13. <i>H-RasV12 and K-RasV12 co-localize with ASPP2 on the cell membrane</i>	222
Figure 3.14. <i>ASPP1 sub-cellular localization changes in the presence of K-RasV12</i>	224
Figure 3.15. <i>H-RasV12 and K-RasV12 activate endogenous ASPP1 and ASPP2</i>	227
Figure 3.16. <i>Construction of plasmids expressing H-Ras RNAi and K-Ras RNAi</i>	230
Figure 3.17. <i>Endogenous Ras is necessary for ASPP2 full transactivation activity</i>	234
Figure 3.18. <i>Endogenous H-Ras and K-Ras can inhibit ASPP1- and ASPP2-induced apoptosis</i>	236
Figure 3.19. <i>Endogenous H-Ras and K-Ras are necessary for cisplatin-induced apoptosis</i>	238
Figure 3.20. <i>Endogenous Ras is involved in ASPP1- and ASPP2-mediated apoptosis in cells treated with cisplatin</i>	240

Chapter 4: Ras stimulates ASPP1 and ASPP2 via the Raf-MEK-MAPK pathway

Figure 4.1. <i>Activated Raf increases ASPP2 activity to a similar extent as oncogenic Ras</i>	255
Figure 4.2. <i>ASPP1 and ASPP2 contain two putative MAPK phosphorylation sites</i>	257
Figure 4.3. <i>ASPP2 is phosphorylated in vitro by MAPK</i>	260
Figure 4.4. <i>S-4 is a phosphospecific antibody that recognizes ASPP2 phosphorylated at serine 827</i>	262
Figure 4.5. <i>ASPP2 is phosphorylated in vivo by MAPK</i>	264
Figure 4.6. <i>Endogenous MAPK is required for full ASPP2 activity</i>	266
Figure 4.7. <i>Construction of plasmids expressing ASPP2 phosphorylation mutants</i>	268
Figure 4.8. <i>ASPP2 (S827A) does not respond to Raf CX stimulation</i>	271

Figure 4.9. <i>ASPP1 phosphorylation mutant S746A does not respond to K-RasV12 stimulation.....</i>	274
Figure 4.10. <i>ASPP2 (S827A) does not synergize with p53.....</i>	276
Figure 4.11. <i>K-RasV12 effect on ASPP1 change of cellular localization is dependent on MAPK activity.....</i>	278
Figure 4.12. <i>ASPP1 does not change its localization in Normal Human Fibroblasts after EGF stimulation.....</i>	280
Figure 4.13. <i>H-RasV12 stabilizes ASPP2 wt but not ASPP2 (S827A).....</i>	283
Figure 4.14. <i>Pulse chase of ASPP2 wt and ASPP2 (S827A) in the presence or absence of K-RasV12.....</i>	285
Figure 4.15. <i>MAPK activity is necessary for endogenous ASPP2 stability.....</i>	287
Figure 4.16. <i>ASPP2 is not necessary for MAPK activation in MEFs.....</i>	289
Figure 4.17. <i>MAPK inhibitor UO126 reduces doxorubicin-induced apoptosis in MCF7 cells.....</i>	291
Figure 4.18. <i>ASPP2 (S827A) phosphorylation mutant co-localizes with K-RasV12....</i>	293

Chapter 5: Regulation of ASPP1 and ASPP2 activity

Figure 5.1. <i>Schematic diagram of mouse and human ASPP1 and ASPP2 deletion fragments</i>	311
Figure 5.2. <i>ASPP2 (1-360) increases p53 activity.....</i>	312
Figure 5.3. <i>The amino-termini of ASPP1 and ASPP2 stimulate p53 and full length ASPP1 and ASPP2 transactivation activity.....</i>	314
Figure 5.4. <i>Human ASPP1(1-310), human ASPP2 (1-360) and mouse ASPP2 (59-383) can increase p53 and ASPP2 transactivation activity.....</i>	316
Figure 5.5. <i>ASPP2 (1-360) stimulates ASPP2 wild type and ASPP2 (S827A).....</i>	318
Figure 5.6. <i>Putative ASPP2 kinases.....</i>	320
Figure 5.7. <i>ASPP2 (693-1128) is phosphorylated in vitro by a variety of kinases.....</i>	322
Figure 5.8. <i>PKA phosphorylates ASPP2 in vitro with high activity.....</i>	324
Figure 5.9. <i>PKA phosphorylates ASPP2 (693-1128) in vitro on residue 737.....</i>	326
Figure 5.10. <i>Construction of plasmids expressing an ASPP2 phosphorylation mutant at the PKA site.....</i>	328

Figure 5.11. *ASPP2 wild type and ASPP2 (S736A,S737A) have similar activity.....*331

Figure 5.12. *The PKA activator forskolin stimulates ASPP2 wild type and ASPP2 (S736,737A).....*334

Chapter 6: Final Summary and Discussion

Figure 6.1. *Model for Ras-ASPP2 negative feedback.....*346

Figure 6.2. *Oncogenic Ras induces apoptosis in an ASPP- and p53-dependent manner.....*350

Figure 6.3. *Ras stimulates p53 to induce cell cycle arrest and apoptosis.....*352

List of tables

Table 1.1. *Dysfunctions of the Rb pathway found in cancers.....*33

Table 1.2. *Summary of p53 binding proteins.....*80

List of Abbreviations

Abbreviation	Full name
53BP	p53 binding protein
Ab	Antibody
ACTH	Adrenocorticotrophic hormone
AIF	Apoptosis inducing factor
APC	Adenomatous polyposis coli protein
APC	Anaphase-promoting complex
ASPP	Apoptosis stimulating protein of p53
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia-mutated protein
ATR	ATM-and Rad-3-related protein
Bbp2	Bcl-2 binding protein 2
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CAD	Caspase-activated DNase
CAK	Cdk activating kinase
CARD	Caspase recruitment domain
CBP	CREB-binding protein (also known as p300)
Cdk	Cyclin-dependent kinases
ChIP	Chromatin immunoprecipitation assay
CHX	Cyclohexamide
CIP 1	CDK inhibitor protein 1
CKI	Cdk kinase inhibitor
CREB	cAMP-response-element-binding protein
CRD	Cysteine-rich domain
DD	Death domain
DED	Death effector domain
DISC	Death-inducing signalling complex
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acids
dNTP	Deoxynucleotides triphosphate
DSB	Double-stranded breaks

ECL	Enhance chemi-luminescence
EEC	Ectrodactyly, ectodermal dysplasia, facial clefts
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FACS	Fluorescence-activated cell sorter
FasL	Fas ligand
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
G1	Gap-1
G2	Gap-2
GAP	GTPase activating protein
GDP	Guanine diphosphate
GNEF	Guanine nucleotide exchange factor
GSK3	Glycogen synthase kinase 3
GTP	Guanine triphosphate
HDAC	Histone deacetylases
HPV	Human papilloma virus
IAP	Inhibitors of apoptosis proteins
IGF-BP3	Insulin-like growth factor binding protein 3
IR	Ionizing radiation
JNK	Jun N-terminal kinase
Kd	Kilodalton
KSR	Kinase suppressor of Ras
LOH	Loss of heterozygosity
M	Mitosis
MAPK	Mitogen-activating protein kinase
MEFs	Mouse embryo fibroblasts
MEK	MAPK extracellular signal-regulated kinase
mRNA	Messenger RNA
MSV	Murine sarcoma viruses
NB	Nuclear body
NES	Nuclear export signal
NF1 disease	von Recklinghausen's neurofibromatosis
NHF	Normal human fibroblasts
NLS	Nuclear localization signal

NP40	Nonipet P-40
p53AIP	p53-regulated apoptosis-inducing protein
p90 ^{RSK}	p90 ribosomal S6 kinase
PAGE	Polyacrilamide gel electrophoresis
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositide-dependent protein kinase 1
PERP	p53 apoptosis effector related to PMP-22
PH	Pleckstrin homology
PI	Propidium iodide
PI3K	Phosphoinositol 3 kinase
PIG	p53-inducible genes
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLD	Phospholipase D
PML	Promyelocytic leukaemia protein
PtdIns[3,4,5]P ₃	Phosphoinositol (3,4,5) triphosphate
PtdIns[4,5]P ₂	Phosphoinositol (4,5) biphosphate
PTP	Permeability transition pore
RA	Ras association domain
RAI	RelA-associated inhibitors
Rb	Retinoblastoma protein
RBD	Ras binding domain
REFs	Rat embryo fibroblasts
Rlu	Relative luminescence units
RNA	Ribonucleic acids
RNAi	RNA interference
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Synthesis
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SCF	Skp1-Cullin1 F-box protein complex
SDS	Sodium dodecyl sulphase
Sos	Son of sevenless
SRE	Serum response elements

ssDNA	Single-stranded DNA
TAFs	TBP-associated factors
TBP	TATA-binding proteins
TNF	Tumour necrosis factor
TNFR	Tumour necrosis family receptors
TSA	Trichostatin A
TSP1	Thrombospondin-1
UTR	Untranslated region
UV	Ultra-violet radiation
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau tumour suppressor protein
WAF1	Wild-type p53 associated fragment 1
WT1	Wilm's tumour 1 protein

Chapter 1

Introduction

1.1 Cancer

The last quarter of a century has seen a significant increase in our understanding of cancer. It is now widely accepted that cancer progression arises as a result of small lesions in our DNA known as somatic mutations, as well as gene silencing by epigenetic changes, such as gene methylation. Mutations occur following a number of different carcinogens such as ultra-violet light, ionizing radiation and reactive oxidative metabolites. Insight into this disease has been enhanced with the recognition that there are two classes of cancer genes: proto-oncogenes and tumour suppressor genes. Proto-oncogenes are commonly mutated in cancers to become oncogenes, with a gain of function relative to their wild-type counterparts. These proteins function by regulating cell cycle proliferation, survival and angiogenesis. Tumour suppressor genes, however, are recessive genes: their function is lost or compromised in tumour cells. Tumour suppressor genes regulate diverse cellular activities including cell cycle checkpoint responses, detection and repair of DNA damage, and differentiation.

Tumour suppressor genes play a crucial role in preventing the formation of tumours and usually a single functional allele is sufficient for their full activity. As such, tumourigenesis requires inactivation of both alleles of the tumour suppressor genes; this is known as the “two-hit” theory (Knudson, 1971). The term “tumour suppressor” includes a wide range of genes involved one way or another in preventing the formation

of tumours. In reality, there are subsets of genes that fit under the category of tumour suppressors.

There are three main sub-groups of tumour suppressor genes as described by Kinzler and Vogelstein (Kinzler and Vogelstein, 1998): the “gatekeepers”, “caretakers” and “landscapers”. The gatekeeper genes represent the tumour suppressors that directly modulate cell proliferation and cell death, such as p53, retinoblastoma protein (Rb), von Hippel-Lindau protein (VHL) and adenomatous polyposis coli protein (APC). They are known as gatekeepers because they prevent “runaway” growth of cells; a mutation of a gatekeeper gene leads to a permanent imbalance of cell division over cell death. Some gatekeeper proteins are specific to a particular tissue, for example retinoblastoma mutations affect retinal epithelial cells, VHL is the gatekeeper to kidney cells and APC the gatekeeper to colon cells. The “caretakers” of the genome are susceptibility genes that indirectly suppress neoplasia, such as XPB, ATM, MSH2 and MLH1. These generally encode DNA repair proteins. Inactivation of a caretaker gene leads to an increased mutation rate, resulting in a higher probability of the cell becoming tumourigenic. The “landscapers” are genes which, when mutated, can increase the risk of an altered stromal environment. This is seen in patients with juvenile polyposis syndromes that have an increased risk of the adjacent epithelia becoming malignant.

There are over one hundred distinct types of cancers and many more subtypes of tumours. Nonetheless, most, if not all, tumours share many similar acquired capabilities, resulting in the cells being defective in regulating proliferation and homeostasis. The vast majority of cancer cells have six essential alterations in their physiology that allow them to achieve malignant growth (Hanahan and Weinberg, 2000). These alterations

result from mutation in oncogenes and tumour suppressor genes; although these mutations can be tissue specific, some are common in many different types of cancers as shown below in brackets. The essential alterations to form cancer cells are: self-sufficiency in growth signals (e.g. activated H-Ras); insensitivity to growth-inhibitory signals (e.g. loss of Rb); evasion of programmed cell death, known as apoptosis (e.g. loss of p53); limitless replicative potential (e.g. activated telomerase); sustained angiogenesis (e.g. VEGF produced); and tissue invasion and metastasis (e.g. inactivation of E-cadherin).

The progressive transformation of normal cells to malignant derivatives follows a multistep process, with each step being equivalent to further genetic alterations. This is exemplified in the colorectal cancer system where the well-defined progression to cancer is a result of at least 7 distinct steps, including mutations of APC, K-Ras, DCC/DPC4 and p53 among others (Kinzler and Vogelstein, 1996). Transformation of cultured cells is also a multistep process as rodent cells need at least two introduced genetic changes to acquire tumourigenic competence (Hahn et al., 1999).

As mentioned above, most changes in cancer cells are acquired through mutations in the genome. However, mutation rates in cells are rare events due to an extensive system of proteins that can monitor DNA damage and repair it. Thus, the genomes of cancer cells must be more prone to mutations than their normal counterparts for the tumour to progress. Indeed, inactivation of genomic gatekeepers at an early stage of the tumours results in increased mutability (Lengauer et al., 1998).

The selection of cancer cells following a series of genetic alterations is highly analogous to Darwin's evolution theory, with genomic changes conferring growth and survival advantages over the non-transformed cells. An oncogenic mutation of a tumour cell allows for clonal expansion of the affected cell, thereby propagating the initial mutation. As cells that have an initial mutation and growth advantage are selected for, they have a higher chance of undergoing more mutations as they proliferate. Thus tumour cells are selected for in a similar manner to evolution's natural selection process. The varying tumours will have different mutations and different growth patterns depending on the stromal environment they are growing in and what part of the body they are in, but all will share the common property of proliferating independently of growth control signals and having reduced sensitivity to apoptosis-inducing signals.

1.2 Cell cycle

In order for a cell to reproduce and give rise to two daughter cells, it must duplicate its content and then divide in two. The process of cell division is known as the cell cycle and is highly conserved from yeast to humans. There are two main phases in the cell cycle: mitosis (M), when the duplicated chromosomes segregate to form two daughter cells, and the interphase which is the time between one round of mitosis and another. The interphase can be separated into three distinct temporal and functional periods: S phase (Synthesis) which is when the cell replicates its DNA content from $2n$ to $4n$; G1 (Gap-1) which is the phase between mitosis and S phase, where most of the proteins and RNAs are synthesised; and G2 (Gap-2) which follows DNA duplication and prepares for the process of division. Another phase, known as G0, is the quiescent phase, when the cell is no longer cycling but is metabolically active. In some cases, cells in G0 can re-enter the cell cycle following the necessary signals.

Progression through the cell cycle is tightly regulated and proceeds in the strict temporal order of G1, S, G2 followed by M to ensure the proper duplication of DNA before separation of chromosomes. In order for the cell cycle to start dividing, it must be of a critical mass and it must respond to external stimuli, such as the presence of nutrients or growth factors.

The cell cycle is regulated by the sequential and periodic activation and inactivation of cyclin-dependent kinases (Cdks). There are 9 known Cdks in mammals and these are generally found at constant levels throughout the cell cycle (Johnson and Walker, 1999). The main mechanism of Cdk regulation is its association with its binding

partners, the cyclins. Although there are 16 known cyclins, only a proportion of those have clearly defined roles in regulating the cell cycle. Cyclins were first identified by Tim Hunt as proteins that had oscillating levels throughout the cell cycle and he proposed that they were somehow involved in cell division (Evans et al., 1983). Indeed, time has proved him right, and they are now known to be key regulators of the cell cycle. The binding of cyclins to their appropriate Cdk partner activates the Cdk, and the complex is then competent to phosphorylate a number of substrates resulting in the progression of the cell cycle (Johnson and Walker, 1999). To maintain the temporal order of the cell cycle, individual Cdk-cyclin pairs must be activated only at specific points. This is ensured by the constant, highly regulated, cycle of synthesis and degradation of cyclins.

There are three main groups of cyclins involved in the cell cycle: the G1 cyclins (D-type cyclins), the S phase cyclins (cyclins E and A) and the mitosis cyclins (cyclins B and A) (Murray, 2004). Entry into the G1 phase from the quiescent G0 phase requires the cyclin D/Cdk4 and cyclin D/Cdk6 partners. These phosphorylate many substrates, resulting in the synthesis of cyclin E. Once synthesised, cyclin E associates with Cdk2 to activate it at late G1 phase. Entry into S phase is dependent on both cyclin E/Cdk2 and cyclin A/Cdk2 activities. As S phase progresses, cyclin A associates with a different Cdk, Cdk1, leading the cell into G2 phase after the successful completion of DNA replication. The entry into mitosis is correlated with the activation of cyclin B/Cdc2 complex (summarized in figure 1A) (Johnson and Walker, 1999).

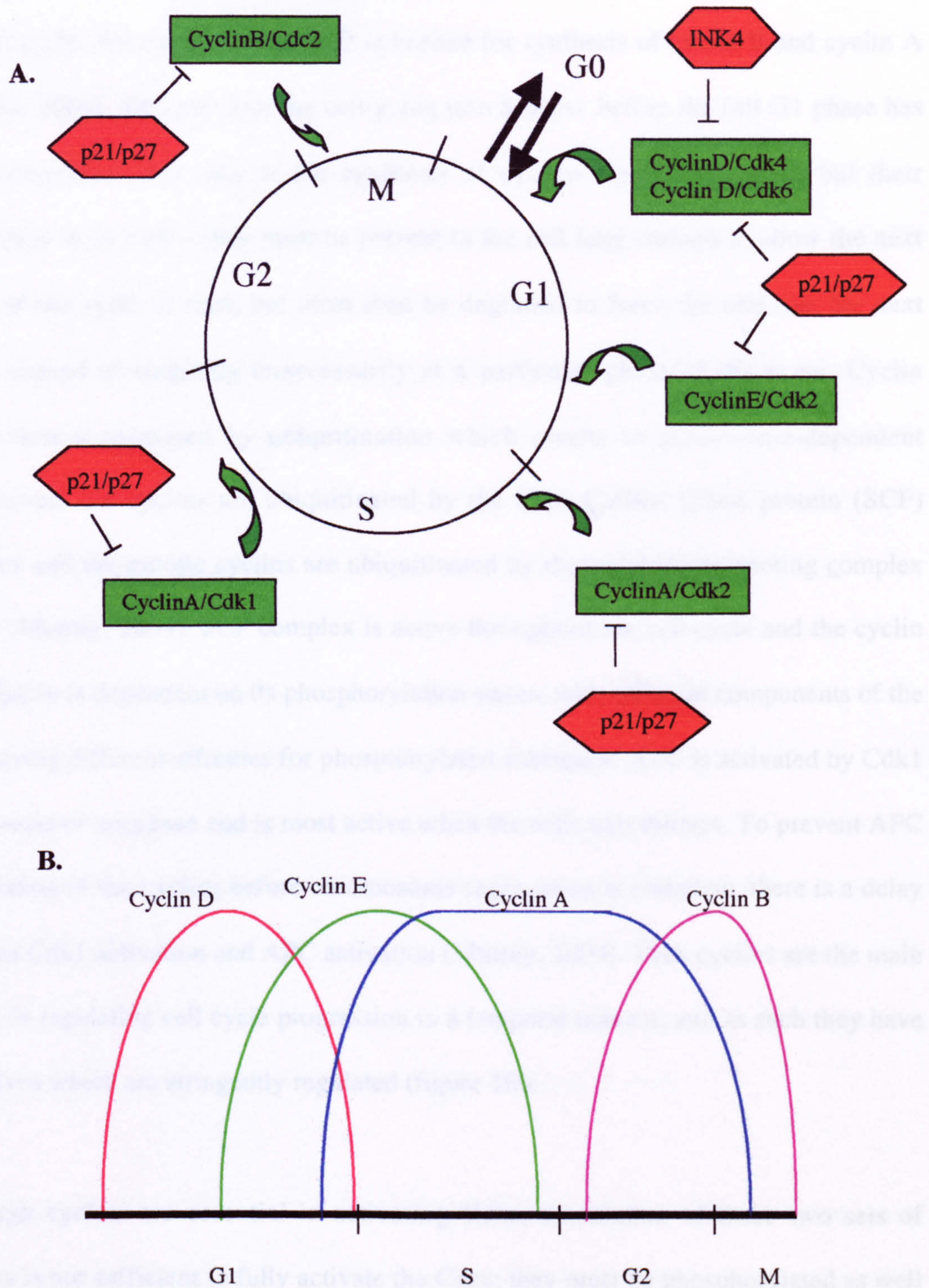


Figure 1.1 Cyclins and the cell cycle. (A) The four successive phases of a standard cell cycle are shown as G1, S, G2 and M. G0 represents withdrawal of the cell cycle. The arrows represent the stage of the cell cycle where the cyclins and their cyclin-dependent kinase partners are active and necessary for progression. p21, p27 and INK4 are cyclin kinase inhibitors which prevent the activity of certain cyclins as shown. (B) The synthesis and degradation of the principle cyclins involved in cell division.

The protein levels of cyclins are tightly regulated to ensure smooth progression through the cell cycle. For example, cyclin D is needed for synthesis of cyclin E and cyclin A (Murray, 2004). This prevents the cell going into S phase before the full G1 phase has been completed. Not only is the synthesis of cyclins tightly regulated, but their destruction is as well – they must be present in the cell long enough to allow the next phase of the cycle to start, but must then be degraded to force the cell into the next phase instead of lingering unnecessarily at a particular phase of the cycle. Cyclin destruction is regulated by ubiquitination which results in proteasome-dependent degradation. G1 cyclins are ubiquitinated by the Skp1-Cullin1 E-box protein (SCF) complex and the mitotic cyclins are ubiquitinated by the anaphase-promoting complex (APC) (Murray, 2004). SCF complex is active throughout the cell cycle and the cyclin degradation is dependent on its phosphorylation status, with different components of the SCF having different affinities for phosphorylated substrates. APC is activated by Cdk1 at the onset of anaphase and is most active when the cells exit mitosis. To prevent APC degradation of the cyclins before chromosome segregation is complete, there is a delay between Cdk1 activation and APC activation (Murray, 2004). Thus cyclins are the main factors in regulating cell cycle progression in a temporal manner, and as such they have short lives which are stringently regulated (figure 1B).

Although cyclins are essential in activating Cdks, association of these two sets of proteins is not sufficient to fully activate the Cdks; they must be phosphorylated as well to achieve their full activation potential. The Cdk activating kinase (CAK) is a common activator of most Cdks and is composed itself of Cdk7 and cyclin H (Murray, 2004). Cdks are inactive when hypophosphorylated, but cyclin binding and threonine

phosphorylation by CAK in a region called the T loop results in an active enzyme complex.

A further level of complexity in Cdk regulation comes from the Cdk-inhibitors (CKIs). There are two families of CKIs: the INK4 family and the CIP/KIP family. The INK4 Cdk inhibitors are made up of 4 members: p15, p16, p18 and p19. These CKIs are specific for Cdk4 and Cdk6 which can only bind the D-type of cyclins. The INK4 family members therefore control the G1 phase of the cell cycle and they do so by competing for the binding of the Cdk4/6 with the D-cyclins (Johnson and Walker, 1999; Pines, 1997; Vidal and Koff, 2000). The other CKI family member, CIP/KIP, is less specific and can inhibit all cyclin-Cdk complexes. The family is composed of three members: p21, p27 and p57 (Vidal and Koff, 2000). The crystal structure of cyclinA-Cdk2-p27 has lead to much insight as to the mechanism of action of p27: p27 was found to bind cyclin A, as well as Cdk2. Binding to Cdk2 eliminated the glycine loop essential for ATP binding and blocked the ATP binding residues in the catalytic cleft, resulting in an inactive Cdk2 (Pines, 1997). Even though the crystal structure was made with incomplete cyclin A and p27, the results matched previous mutagenesis data suggesting that the interactions seen in the crystal structure also occur *in vivo*.

As well as being regulated by synthesis and destruction of cyclins, phosphorylation and CKIs, cyclin-dependent kinases are also regulated by the sub-cellular localization of the cyclin-Cdk complexes. Whereas some cyclins are invariably nuclear (cyclin A), and others are invariably seen to be cytoplasmic (cyclin B2), others have been shown to shuttle in and out of the nucleus. Cyclin D1 is nuclear in G1 but as S phase begins, it is exported to the cytoplasm following phosphorylation, where it is degraded (Yang and

Kornbluth, 1999). On the other hand, cyclin B1 is synthesised in S phase in the cytoplasm and once cells enter prophase, it translocates to the nucleus where it is active in regulating Cdc2 (Yang and Kornbluth, 1999).

There are two major points in the cell cycle where the decision could be made to proceed with the division or to pause, and these are known as checkpoints. Checkpoints represent control stages where the initiation of the next phase of the cycle is dependent on the successful completion of the previous stage. The first checkpoint is present in G1 and is known as the “restriction point” in mammals, and “start” in yeast. Once the restriction point has passed, the cell can no longer delay the entry into S phase and therefore proceeds with the cell cycle until the completion of mitosis, barring severe DNA damage or metabolic distress. The second checkpoint is the G2/M checkpoint that prevents entry into mitosis following incomplete DNA replication. The delay into the M phase allows the DNA to be successfully duplicated before chromosomes are separated. The main regulator of the G1 restriction point is the retinoblastoma protein, whereas p53 can regulate entry into various stages of the cell cycle in response to stress signals.

1.2.1. Rb regulation of the cell cycle

Up until the restriction point in late G1, the decision of the cell to cycle is dependent on external stimuli. Past this point, external stimulus no longer can affect the decision of the cell to cycle and the cell undergoes a full round of division. The retinoblastoma (Rb) protein is thought to be crucial for this checkpoint (Bartek and Lukas, 2001). Before the restriction point, Rb is hypophosphorylated in cells, whereas beyond this checkpoint it is hyperphosphorylated. It has therefore been suggested that phosphorylation of Rb causes it to open the gates of cell division, allowing the cell to continue through its cycle (Planas-Silva and Weinberg, 1997).

The understanding of Rb function has been enhanced by the discovery of its binding partners. Whilst Rb is known to bind a number of different proteins such as Elf-1, MyoD and c-Abl, it seems that its main function is to regulate E2F proteins by associating with them (Weinberg, 1995). The E2F family is composed of 6 members; three of the E2F proteins, E2F1-3, associate with Rb and two others, E2F4 and E2F5, associate with the Rb family members p107 and p130. All of the E2F members, except for E2F6, are transcription factors. In cells, the E2F proteins are found as heterodimers, associated with one of the two DP family members, DP1 or DP2. DP and E2F activate E2F-dependent transcription in a synergistic manner (Dyson, 1998).

E2F1 is thought to be the main substrate of Rb since ectopically expressed E2F1 is sufficient to lead the cell from the G0 quiescent phase to the S phase of the cell cycle (Johnson et al., 1993). Thus E2F1 can lead the cell into the cell cycle and the main function of Rb is to restrict cell cycle progression by negatively regulating E2F activity, until the proper signal has been sent. This was confirmed by overexpression of Rb

inhibiting E2F-dependent transcription (Flemington et al., 1993; Helin et al., 1993; Hiebert et al., 1992).

Since Rb is regulated by phosphorylation, it came as no surprise to find that the phosphorylation status of Rb influenced its regulation of E2F. Indeed, hypophosphorylated Rb is strongly associated with E2F, whilst hyperphosphorylated Rb is unable to bind E2F efficiently. Thus, before the restriction point, Rb is associated with E2F, inhibiting its activity, whereas after the restriction point Rb can no longer bind E2F and E2F is therefore free to transactivate a number of genes that can direct the cell further along the cycle (Dyson, 1998).

The region of E2F that binds Rb is situated in its transactivation domain. It was therefore proposed that Rb inhibits E2F activation by physically blocking the action of the transactivation domain (Dyson, 1998). Although this is still thought to be true in some cases, a more complex mechanism for Rb regulation of E2F activity is now emerging. As well as passively inhibiting E2F by blocking its transactivation domain, the Rb-E2F complex can also actively repress a number of genes. This repression has been shown to be mediated by other proteins, such as hBrm and BRG1, and histone deacetylase activity has been associated with Rb, suggesting that E2F-Rb might repress transcription through changes in the chromatin structure (Brehm et al., 1998; Dyson, 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998).

In order for the cell cycle to progress, Rb must be active only in a defined window of time. Following mitogenic stimuli, E2F is released from Rb and initiates the progression of the cell cycle. Rb regulation is at two levels: it is first phosphorylated by cyclin

D/Cdk4 and cyclin D/Cdk6, which are themselves regulated by extracellular stimuli, thus ensuring a direct correlation between these signals and Rb activity. Secondly, following Rb phosphorylation being initiated by cyclin D, Rb is then further phosphorylated by cyclin E/Cdk2, leading to the dissociation of Rb from E2F (Dyson, 1998; Weinberg, 1995). The dissociation of the complex results in the abrogation of the active repression of the E2F-Rb complex, and in the transactivation of E2F target genes. Both these mechanisms lead to a change in overall cellular transcription, resulting in the cell progressing to the next phase of the cell cycle. For example, Rb phosphorylation by cyclin D/Cdk4/6 de-represses cyclin E transactivation, leading to an increase in its transcription. Cyclin E then associates with Cdk2 to further phosphorylate Rb, resulting in the dissociation of Rb from E2F. E2F1 is then free to transactivate cyclin A which can associate with Cdk2, leading the cell into S phase (Dyson, 1998).

Whilst E2F activity is necessary for cells to proceed into S phase, a decrease in E2F activity is required for cells to exit S phase. Following a negative feedback loop, cyclin A has been shown to associate with E2F1/DP, phosphorylate it and inhibit its DNA-binding activity, rendering it inactive (Dyson, 1998). Towards the end of the S phase, E2F1 is targeted for a ubiquitin-proteasome dependent degradation. Thus the cell is then free to proceed into the G2 phase, with low levels of E2F and high levels of cyclin A.

Rb is able to sense a range of different types of upstream influences, from growth-promoting signals and growth inhibitory signals (such as TGF β) to contact inhibition. Negative regulation of Rb is generally mediated by the CKIs which prevent Rb phosphorylation and thus blocking cell cycle progression (Bartek et al., 1997). The

main CKIs involved in Rb regulation are p27 from the CIP/KIP family, and p15 and p16 from the INK4 family. These inhibitors prevent Rb phosphorylation by inhibiting cyclin D/Cdk activity.

Thus Rb plays a central role in integrating internal and external signals and allowing the cell to cycle only under the appropriate conditions. The importance of the Rb pathway in regulating cell cycle is highlighted by the common disruption of the Rb pathway in human tumours. The Rb pathway is rendered dysfunctional in many cancers through mutations and amplifications of its various members (see table 1) and is inactivated by a number of different viral proteins, such as the adenovirus E1A protein, the human papillomavirus (HPV) E7 protein and the SV40 large T antigen.

<i>Member of the Rb pathway</i>	<i>Dysfunction</i>	<i>Type of cancer</i>
Rb	Mutation / <i>SV40</i> <i>HPV</i>	Retinoblastomas Small cell lung carcinoma Sarcomas Bladder carcinomas Cervical carcinomas
Cyclin D	amplification	Esophageal carcinomas Breast carcinomas Squamous cell carcinomas
CDK4	amplification	Glioblastomas Gliomas
p15 or p16	deletion/ silencing due to epigenetic modifications	Esophageal squamous cell carcinomas Glioblastomas Lung carcinomas Bladder carcinomas Pancreatic carcinomas

Table 1. Dysfunctions of the Rb pathway found in cancers. HPV stands for human papilloma virus and SV40 for simian virus 40. Viruses are shown in italic. The most common epigenetic modification of p15 and p16 is methylation of their promoters.

1.2.2. p53 regulation of the cell cycle

Once the cell has passed the restriction point in G1, it is committed to a full round of division, ending after mitosis. As described above, this restriction point is governed by external stimuli that converge on Rb regulation: once Rb function is inactivated the cell will cycle. However, following DNA damage or oncogenic stress, the cell will nonetheless be able to arrest in order to avoid replication and therefore reduce the propagation of genetically damaged cells. This additional checkpoint is governed mainly by p53. p53 is a tumour suppressor protein which is activated in response to various DNA damaging agents such as ultra-violet light and ionizing radiation, as well as many other types of stress. Genotoxic stress leads to post-translational modifications of the p53 protein, resulting in its increased stability following which it is transcriptionally active (Ko and Prives, 1996). p53 can activate a number of target genes, many of which are involved in cell cycle arrest (see section 1.4.2).

The best-studied target of p53 is p21, also known as CIP or WAF-1. p21 was discovered simultaneously by two separate groups; one group found that it was a potent inhibitor of the cyclin-dependent kinases and called it CIP1 for Cdk-inhibitor protein-1, and the other group that it was a novel p53 effector protein and called it Waf1 for wild-type p53 associated fragment-1 (El-Deiry et al., 1993; Harper et al., 1993). For simplicity, I shall call it p21 from hereon. p53 was found to induce p21 which in turn could inhibit all the cyclin-Cdk complexes involved in the regulation of the cell cycle (Johnson and Walker, 1999; Vidal and Koff, 2000). Once the early Cdks are inhibited, particularly the cyclinD/Cdk4 and cyclin E/Cdk2, Rb can no longer be phosphorylated, following which E2F is no longer released to induce the cell cycle. Unlike the Rb restriction point, however, p21 can induce cell cycle arrest throughout G1, even in late

G1, due to its capacity to inhibit cyclin A/Cdk2 which is necessary for progression into S phase. Although the main role of p21 is to induce cell cycle arrest at the G1/S boundary of the cycle, it has also been shown to induce arrest at the G2 phase of the cell cycle. This is due to its capacity to inhibit the activity of all Cdks, including cyclin B/Cdc2.

As well as inducing cell cycle arrest, p21 has another role: it can inhibit activation of polymerase δ , which allows chromosomal DNA replication, by the proliferating cell nuclear antigen (PCNA) (Kelman, 1997). Thus, inhibition of PCNA by p21 results in a decrease in DNA replication. This decrease in the rate of DNA synthesis would allow the DNA damage to be repaired before it is replicated, preventing propagation of the damage.

As well as *p21*, p53 targets other genes involved in cell cycle arrest: *GADD45* is involved in the G1/S checkpoint, whereas *14-3-3 σ* and *reprim* can arrest cells in G2 (Hermeking et al., 1997; Kastan et al., 1992b; Ohki et al., 2000). Yet another p53 target gene is *p53R2*, a nucleotide reductase subunit known to be involved in DNA repair (Tanaka et al., 2000). This confirms a role for p53 in allowing the cell to overcome DNA damage. Interestingly, p53 triggers cell cycle arrest following only low levels of DNA damage; if the DNA damage levels are beyond a particular threshold, p53 will then induce apoptosis.

1.3 Apoptosis

In order for a homeostatic state to occur in a multicellular organism, the context and number of cells interacting with each other must be strictly regulated. This can be achieved by regulating the cell cycle, and thus the proliferation of a cell, as well as by controlling the process of cell death.

There are two types of cell death: necrosis and apoptosis. Whereas necrosis has been understood for a long time, apoptosis is a relatively new field, only discovered in the last 30 years or so. The rising awareness of a different type of cell death from necrosis started with microscopic observations of cells. Whilst looking at ischaemic liver injuries, John Kerr noticed some small round masses of cytoplasm that contained condensed chromatin and these cells were morphologically distinct from cells undergoing necrosis (Kerr, 1965). The main differences he observed with these cells compared to necrotic cells were their histological appearance; the fact that only a few isolated cells were affected; that there was no inflammation; and that they were non-degenerative with no lysosomal leakage. Further work by electron microscopy showed a clear morphological phenotype: condensation of the cytoplasm, aggregation of compacted chromatin beneath the nuclear envelope, small round bodies containing fragments of nucleus and condensed cytoplasm, and plasma membrane blebbing whilst the organelles were preserved (Kerr, 1969; Kerr, 1971). He named this process “shrinking necrosis”. Cells undergoing this process were seen to be engulfed by specialized phagocytes which then proceeded to digest them.

During this time, two other pioneers of the field, Andrew Wyllie and Alistair Currie also saw similar processes by light microscopy, looking in different tissues and species. Wyllie observed that this “shrinking necrosis” also occurred in adrenal cortices of rats following treatment with a suppressor of adrenocorticotrophic hormone (ACTH), suggesting that this process could be regulated by hormones (Wyllie et al., 1973a). Further work showed that the “shrinking necrosis” phenotype could be found in normal neonatal rats, following a physiological fall in ACTH, suggesting for the first time that this could be a naturally occurring process in development (Wyllie et al., 1973b). Confirming the role of hormone regulation in this phenotype, injection of ACTH prevented cell death. This same phenotype of cell death was also seen to occur during regression of rat breast carcinomas after ovary removal (Kerr and Searle, 1972).

Following this ground-breaking work, Kerr, Wyllie and Currie introduced the concept of apoptosis as a distinctive, inherently programmed, cell death (Kerr et al., 1972). The term apoptosis correlated with the notion of cell suicide: the cell induces and actively participates in its own death. As originally suggested, apoptosis was found to occur in various different tissues and species and remains a tightly regulated process in normal development and adult cells. Inactivation of apoptosis has since been associated with cancer development and autoimmune diseases whereas its aberrant activation is associated with neurodegenerative diseases and stroke.

Following the discovery of a conserved molecular machinery that mediates apoptosis, the morphological definition has slowly been replaced by a functional one. Indeed, although signalling for apoptosis occurs through multiple independent pathways that

can be triggered by either intracellular or extracellular events, all signalling pathways converge on caspase activation which ultimately leads to cell destruction.

1.3.1. Caspases

There are 15 known human caspases, of which seven are known to be involved in apoptosis. Caspases are cysteine proteases that cleave their substrates at the carboxy terminal side of an aspartate residue. Although all caspases recognize tetrapeptide motifs, each caspase has its own substrate specificity (Strasser et al., 2000). Caspases are produced as zymogens, with their catalytic site inactive. Following stimulation by upstream regulators, they are sequentially cleaved, first with the removal of the amino-terminal prodomain, followed by a further cleavage resulting in a large and a small subunit which associate to form an active enzyme (Danial and Korsmeyer, 2004). Active caspases are found as tetramers in cells, composed of two heterodimers, thus containing two active sites.

There are two types of caspases: the initiators (caspase 8 and caspase 9) and the effectors (all other caspases). Proteolysis and activation of the initiator caspases results in a downstream cascade of caspase activation. Caspases are thought to have up to 60 different substrates, all involved in the processing of apoptosis, ranging from cytoskeletal and structural proteins, proteins involved in cell cycle and replication (e.g. Rb), transcription factors (e.g. NF- κ B), protein kinases (PKC) and Bcl2 family members (e.g. Bid) (Nagata, 1999). One invariable feature of apoptosis is the degradation of chromosomal DNA which was also shown to be mediated by caspases. Caspase 3 and caspase 7 cleave iCAD, an inhibitor of the caspase-activated DNase

(CAD). Proteolysis of iCAD results in its degradation, following which the active CAD is free to cleave chromosomal DNA (Nagata, 1999). DNA cleavage may occur to prevent spreading of cancerous genomes or viral DNA to the phagocytes that digest the apoptotic cells.

1.3.2. Regulation of caspases through the death receptor pathway

Apoptosis can be triggered in some cells by extracellular signals, such as cytokines. These signals are sensed by transmembrane receptors, known as death receptors, that have similarity to the tumour necrosis family receptors (TNFR). This family of death receptors includes the Fas/CD95, TNFR1, DR-3/TRAMP, DR4/TRAIL-R1 and DR5/KILLER receptors (Budihardjo et al., 1999). One well studied death receptor pathway is the FasL-Fas pathway. The Fas ligand (FasL) makes contact with the receptor Fas, following which Fas forms a trimer. The homotrimer of Fas can then recruit the cytosolic adapter protein FADD through interactions of death domains (DD) found in both the receptor and the adaptor protein. The complex of the Fas trimeric receptor and adaptor protein is known as the death-inducing signalling complex (DISC) (Budihardjo et al., 1999; Nagata, 1999). This complex can then recruit the initiator procaspase 8 by the interaction of the death effector domain (DED) found in both the FADD adaptor protein and procaspase-8. Once procaspase 8 is bound to the DISC, it oligomerizes, resulting in the cleavage of procaspase 8 to the enzymatically active caspase 8 (Budihardjo et al., 1999; Nagata, 1999). Caspase 8 then cleaves a number of downstream targets, including other caspases, as well as the pro-apoptotic protein Bid (figure 1.2). Cleavage of bid activates it and it is then translocated to the mitochondria, stimulating the mitochondrial-apoptotic pathway (Budihardjo et al., 1999; Danial and

Korsmeyer, 2004). It is worth noting that in some cases activation of the death receptor pathways is sufficient to induce apoptosis in a manner that is independent of the mitochondrial pathways but in other cases it is necessary for the death receptor pathway to activate the mitochondrial pathway to induce a full apoptotic response. It is not yet fully understood when the mitochondrial pathway is necessary for the death receptor pathway to induce apoptosis.

Death receptor pathways can be negatively regulated in many ways. The first manner by which activation of the pathway is prevented, is by preventing procaspase 8 recruitment to the death receptor. This is done, for example, by the viral protein vFLIP that has two DED domains, thereby competing with procaspase 8 binding to the receptor. A mammalian homolog, cFLIP also inhibits the activation of procaspase 8 by the same mechanism (Budihardjo et al., 1999). Another way to negatively regulate the death receptor pathway is through the expression of decoy receptors. These decoy receptors sequester the cytokines, thereby preventing activation of the death receptors. This form of negative regulation of the apoptotic pathway is especially prominent in lung and colon carcinomas, where more than 50% of these carcinomas have amplified and overexpressed the DcR3 decoy receptor to FasL (Budihardjo et al., 1999; Nagata, 1999). One other way to inhibit the death receptor pathway is to inhibit the proteolytic activation of procaspase 8, which is the mechanism of action of the viral protein CrmA (Budihardjo et al., 1999).

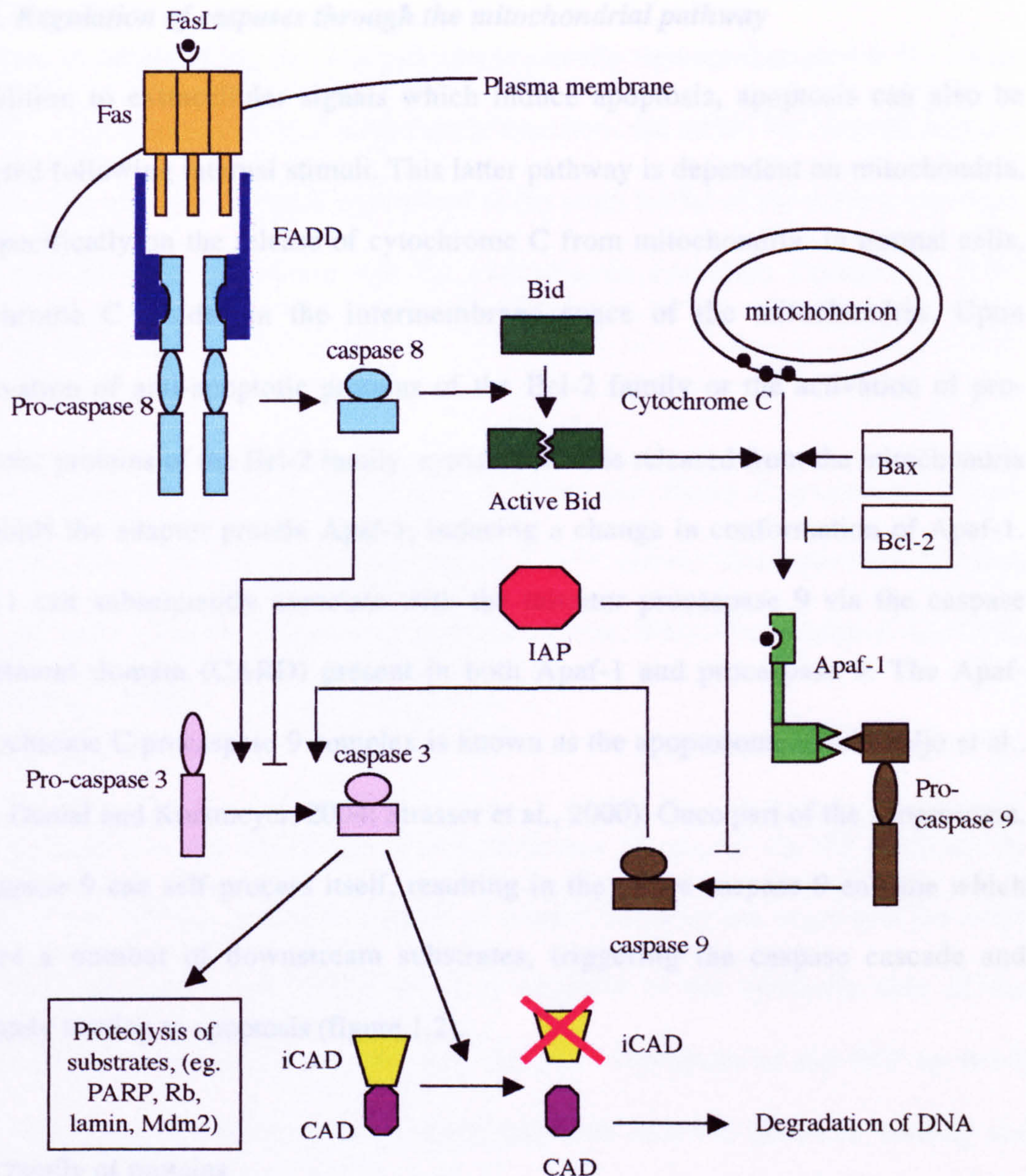


Figure 1.2 *Caspase activation via multiple pathways.* The FasL cytokine activates the Fas death receptor by binding to its extracellular region. Activated Fas forms trimers which recruit the FADD adapter protein, forming the death-inducing signalling complex (DISC). The DISC recruits pro-caspase 8 via its DED domain and procaspase 8 is consequently cleaved and activated. Caspase 8 activates procaspase 3 and Bid. Activated Bid can release cytochrome c from the mitochondria, as can Bax. Released cytochrome c associates with Apaf-1, thereby recruiting procaspase 9 to the apoptosome. Procaspase 9 is then cleaved and activated and can activate a number of downstream substrates, including procaspase 3. Activated caspase 3 cleaves a number of substrates, including the CAD inhibitor iCAD. Once CAD is no longer inhibited by iCAD its DNase activity is restored and it can degrade chromosomal DNA. The inhibitor of apoptosis proteins (IAP) can inhibit caspase 3 and caspase 9 activation.

1.3.3. Regulation of caspases through the mitochondrial pathway

In addition to extracellular signals which induce apoptosis, apoptosis can also be triggered following internal stimuli. This latter pathway is dependent on mitochondria, and specifically on the release of cytochrome C from mitochondria. In normal cells, cytochrome C resides in the intermembrane space of the mitochondria. Upon inactivation of anti-apoptotic proteins of the Bcl-2 family or the activation of pro-apoptotic proteins of the Bcl-2 family, cytochrome C is released from the mitochondria and binds the adaptor protein Apaf-1, inducing a change in conformation of Apaf-1. Apaf-1 can subsequently associate with the initiator procaspase 9 via the caspase recruitment domain (CARD) present in both Apaf-1 and procaspase 9. The Apaf-1/cytochrome C/procaspase 9 complex is known as the apoptosome (Budihardjo et al., 1999; Danial and Korsmeyer, 2004; Strasser et al., 2000). Once part of the apoptosome, procaspase 9 can self-process itself, resulting in the active caspase 9 enzyme which cleaves a number of downstream substrates, triggering the caspase cascade and ultimately leading to apoptosis (figure 1.2).

Bcl-2 family of proteins

The Bcl-2 family of proteins is a large family with all members sharing regions of sequence homology, known as BH domains. The number of domains can vary, depending on the proteins, from 4 homology domains (BH1-BH4) to a single domain (BH3). All members of the Bcl-2 family are involved in apoptosis regulation and can be divided into three groups: the anti-apoptotic family members (e.g. Bcl-2, Bcl-X_L, MCL1, Bcl-w) which have all four conserved BH domains; the multi-domain pro-apoptotic members (e.g. Bax, Bak) that have up to three conserved domains; and the pro-apoptotic BH3-only proteins (e.g. Bid), which, as their name suggests, contain only

a single BH3 domain (Danial and Korsmeyer, 2004). The pro-apoptotic and anti-apoptotic members of the Bcl-2 family can physically interact (Strasser et al., 2000). The sub-cellular localization of these family members can differ, for example inactive Bax is in the cytosol and Bcl-2 is localized at the outer leaflet of the nuclear envelope, outer mitochondrial membrane and the endoplasmic reticulum (Antonsson, 2001; Strasser et al., 2000).

The mechanism by which the Bcl-2 family regulates the release of cytochrome C from the mitochondria is still poorly understood. I will discuss three possible theories, although many more have been proposed (Antonsson, 2001; Budihardjo et al., 1999; Strasser et al., 2000).

One hypothesis is that Bcl-2 regulates cytochrome C release via the permeability transition pore (PTP), which is a point of contact between the inner and the outer mitochondrial membranes. Since Bcl-2 is attached to the cytosolic side of the mitochondrial membrane, it may regulate the PTP. Disruption of the PTP by Bcl-2 could lead to a disruption in the electrostatic and osmotic gradient, leading the mitochondrion to swell and rupture, releasing cytochrome C and other pro-apoptotic proteins present in the mitochondria such as the apoptosis inducing factor (AIF).

Another theory is that Bcl-2 can act as an ion channel. The three dimensional structure of the Bcl-2 family member Bcl-xL suggests that it might have an ion channel activity. Anti-apoptotic Bcl-xL is predicted to form cation-specific channels, whereas the pro-apoptotic Bax family member would form anion-selective channels. If the ratios of the anionic and cationic channels are constant, the mitochondrion will have a normal ionic

gradient. However, an increase in pro-apoptotic Bax or a decrease in anti-apoptotic Bcl-X_L would lead to a change in the electrostatic gradient and an increase of water in the mitochondrion, and resulting in the swelling and rupture of the organelle. As a consequence, cytochrome C would be released.

A third possibility for Bcl-2 regulation of the mitochondria is the BH3-containing model. Proteins such as Bid do not contain any other BH domains and are therefore unlikely to form pores. Nonetheless, they are the most potent inducers of apoptosis. Their ability to associate with anti-apoptotic Bcl-2 is directly related to their ability to release cytochrome C from the mitochondria. The mechanism of action of these BH3-only pro-apoptotic proteins is not yet understood.

Since the Bcl-2 family members are so intricately involved in the downstream processing of apoptosis, it is expected that these proteins would be stringently regulated. There are two recognized forms of regulation for the Bcl-2 family members: transcriptional and translational regulation. One obvious example of transcriptional regulation is the chromosomal translocation of the Bcl-2 gene, resulting in the upregulation of its expression with a consequence of inhibiting apoptosis in B cell lymphomas (Antonsson, 2001; Danial and Korsmeyer, 2004). Several pro-apoptotic members of the Bcl-2 family, such as Bax, PUMA and Noxa, have been shown to be downregulated in many tumours. This downregulation of expression is a direct consequence of mutations and/or deletion of their upstream regulator, p53 (Miyashita and Reed, 1995; Nakano and Vousden, 2001; Oda et al., 2000a; Yu et al., 2001). There are several post-translational modes of regulation for the Bcl-2 family (Antonsson,

2001; Danial and Korsmeyer, 2004; Strasser et al., 2000). The pro-apoptotic BH3-only protein Bid is activated by proteolysis following caspase 8 activation. Phosphorylation is also a common modification, regulating the activities of Bad, Bik and Bid. Some of the pro-apoptotic Bcl-2 family members have their activity regulated by their subcellular localization. For example, Bax is in the cytosol when inactive and is found at the mitochondrial membrane when active. Similarly, Bim is bound to the microtubulin-associated dynein complex and is released from this complex upon activation.

1.3.4. Inhibitors of apoptosis proteins (IAP)

There are five known human inhibitors of apoptosis proteins (IAP) and they all share a common motif called BIR. Overexpression of these proteins leads the cells to becoming resistant to various apoptotic stimuli. These proteins can inhibit apoptosis by three different mechanisms. They can interfere directly with the catalytic activity of certain caspases (e.g. caspases 3 and 7); they can prevent the processing of procaspases to caspases, as seen with caspase 9; and they can compete with Apaf-1 binding to caspase 9 by their own CARD domains (Budihardjo et al., 1999). These proteins can therefore inhibit both major pathways of apoptosis.

1.4. p53

Following the discovery of p53 as a binding protein to the SV40 T large antigen, much research has been done on this protein (Lane and Crawford, 1979b; Linzer and Levine, 1979). The importance of p53 as a tumour suppressor protein culminated with the finding that it was the most commonly mutated tumour suppressor protein, with mutations in over 50% of cancers (Hollstein et al., 1994). The crucial role that p53 plays in preventing tumourigenesis was confirmed following the discovery that patients suffering from the Li-Fraumeni syndrome, a hereditary syndrome that predisposes individuals to tumour formation, commonly have germline mutations in the *p53* gene. Correlating with these observations, *p53*-null mice develop normally but are highly predisposed to tumours (Donehower et al., 1992).

A consensus has emerged that p53 can respond to a variety of different stimuli such as DNA damage, oncogenic stress, hypoxia or telomeric erosion. Activation of p53 by these signals results in one of a number of responses ranging from cell cycle arrest and DNA repair to apoptosis. The growing understanding of the role p53 plays in preventing tumour formation has led this protein to be named the “guardian of the genome” (Lane, 1992).

1.4.1. p53: structure-function relationship

As with most transcription factors, p53 is a modular protein with several regions of distinct but inter-dependent functions.

Transactivation domain

The acidic amino terminal region, which spans from residues 1-43, acts as a transcription activation domain and can associate with a number of different proteins. It can recruit basal transcriptional machinery, by interaction with transcription factors such as the TATA-box binding proteins (TBP) and the TBP-associated factors (TAFs) TAF_{II}70 and TAF_{II}31, both subunits of TFIID, thereby activating target genes (Lu and Levine, 1995; Thut et al., 1995). TBP and TFIID have been shown to cooperate with p53 in binding DNA (Chen et al., 1993). The amino terminal domain of p53 can also interact with other proteins such as the p53 negative regulatory factor Mdm2, as well as with TFIID subunits. This latter interaction suggests a role for p53 in transcription/repair.

Residues F19, L22 and W23 are required for transcriptional activation *in vivo*. Mutations in residues 22 and 23 render p53 transcriptionally inactive (Lin et al., 1994) and disrupt its interaction with TAFs, although p53 retains its ability to interact with TBP (Thut et al., 1995).

As well as being a transcription activation site, some studies suggest that the amino-terminal region of p53 can also act as a repressor of transcription. In agreement with this, p53 protein containing mutations in residues 22 and 23 can no longer act as a

transrepressor (Farmer et al., 1996; Sabbatini et al., 1995b), suggesting that TAFs are necessary for p53-mediated repression.

The transcription activation site of p53 is negatively regulated by a variety of proteins. The viral and cellular oncoproteins, E1B and Mdm2, respectively, bind to residues 22 and 23, thereby inhibiting p53 association with the transcriptional machinery. The crystal structure of Mdm2 with a p53 peptide showed that the F19, L22 and W23 residues of p53 stabilize the hydrophobic interactions between p53 and Mdm2 (Kussie et al., 1996); mutations of residues 22 and 23 disrupt the Mdm2-p53 interaction (Lin et al., 1994).

Proline-rich domain

The proline-rich domain (residues 64-91) of p53 has 5 repeats of the PXXP motif and can interact with a number of different proteins through their SH3 domain, such as c-Abl. p53 interaction with c-Abl has been shown to enhance p53 activity (Goga et al., 1995). Deletion of this proline-rich region permits normal p53-mediated transcriptional activation but impairs p53 ability to inhibit tumour cell growth in culture (Walker and Levine, 1996).

DNA binding domain

The core domain of p53 ranges from residue 102 to 292. This region interacts with DNA in a sequence-specific manner (Bargonetti et al., 1993; Halazonetis and Kandil, 1993; Pavletich et al., 1993; Wang et al., 1993). The three dimensional crystal structure of the p53 DNA binding region has enhanced our understanding of how the protein structure is related to its function (Cho et al., 1994). The four conserved regions within

the core domain (boxes II, III, IV and V as shown in figure 1.3) comprise the elements responsible for contacting the major and minor grooves of DNA, whereas the less conserved regions form a scaffold to support the DNA binding elements.

Combining p53 structural studies with mutational analysis helped reveal the effect of mutations on p53 function (Cho et al., 1994). Over 90% of missense mutations occur in the DNA-binding region, highlighting the crucial role this domain has in p53 function. The four highly conserved regions have a particularly high incidence of mutations (Hollstein et al., 1994) and the residues that are most frequently mutated are known as the “hotspots” (figure 1.3). Each of the hotspot residues is found to make a critical contribution to the sequence-specific DNA binding. Other mutations that occur commonly affect either the scaffold or the structural element of the DNA-binding domain and have therefore been classified as contact or conformational mutants.

The tetrameric p53 binds to four repeats of the consensus sequence: 5'-PuPuPuC(A/T)-3' (El-Deiry et al., 1992). This sequence is repeated in two pairs, each arranged as inverted repeats as shown: $\rightarrow\leftarrow\rightarrow\leftarrow$, with each arrow being a consensus sequence. The two pairs can be separated by 0-13 base pairs (El-Deiry et al., 1992).

Linker Region

The flexible linker region (300-320) connects the sequence-specific DNA binding domain to the tetramerization domain.

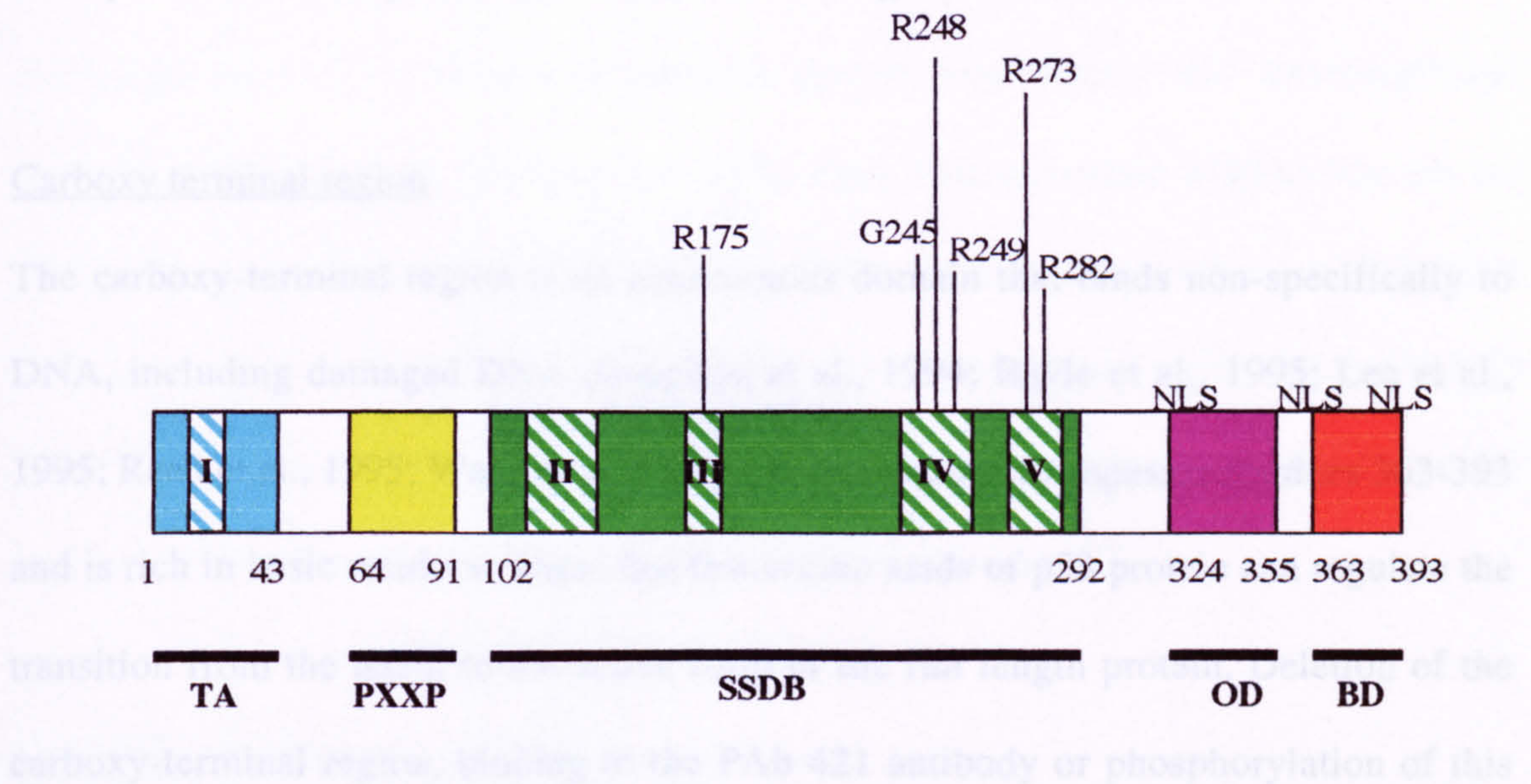


Figure 1.3 *p53 structure*. p53 domains are shown: transactivation domain (TA), proline-rich domain (PXXP), sequence-specific DNA binding domain (SSDB), oligomerization domain (OD) and basic domain (BD). The conserved regions are marked as box I (13-19), box II (117-142), box III (171-181), box IV (234-258) and box V (270-286). Six hotspots are shown representing residues R175, G254, R248, R249, R272 and R282 in human. The length of the vertical line is correlated to the frequency of the residue mutation. The nuclear localization domains are annotated as NLS and are found in the carboxy-terminus of the protein.

Oligomerization domain

p53 is found to be in a tetrameric state in solution, as a dimer of a dimer. The oligomerization domain resides in residues 321-362. The loss of the oligomerization domain results in loss of cell cycle arrest but not growth suppression as measured by colony formation assays (Ishioka et al., 1995; Pellegata et al., 1995).

Carboxy terminal region

The carboxy-terminal region is an autonomous domain that binds non-specifically to DNA, including damaged DNA (Bakalkin et al., 1994; Bayle et al., 1995; Lee et al., 1995; Reed et al., 1995; Wang et al., 1993). This domain encompasses residues 363-393 and is rich in basic residues. These last few amino acids of p53 protein can regulate the transition from the latent to the active form of the full length protein. Deletion of the carboxy-terminal region, binding to the PAb 421 antibody or phosphorylation of this region by CKII or PKC can stimulate p53 to specifically bind to DNA by its central core domain (Halazonetis et al., 1993; Hupp et al., 1992; Takenaka et al., 1995). This region is therefore thought to act as an autoinhibitory domain.

The carboxy-terminal region is believed to encompass a repression domain that can also interact with TBP (Haupt et al., 1995; Horikoshi et al., 1995; Subler et al., 1994). In addition, this domain contains three nuclear localization signals that allow p53 to shuttle from the cytoplasm to the nucleus (figure 3.1).

Domains needed to suppress transformation

p53 mutants lacking the activation domain or the carboxy-terminal region can still suppress transformation, although at a lower frequency than wild-type p53 (Shaulian et

al., 1995; Unger et al., 1993). Growth suppression, however, requires both the amino and the carboxy terminus regions of p53 (Pietenpol et al., 1994). Some mutants can suppress growth but are no longer able to suppress transformation (Crook et al., 1994; Pietenpol et al., 1994) suggesting two separate functions of p53.

Although there is a correlation between growth suppression and transcriptional activation of p53, some p53 mutants retain their transactivation activity but are no longer able to suppress growth (Crook et al., 1994). A p53 protein with a mutation in its transactivation domain rendering it inactive, has defects in cell cycle regulation and apoptosis stimulation (Jimenez et al., 2000). This suggests an essential role of the transactivation domain in the tumour suppressor activities of p53

p53 protein variants

Although *p53* is predominantly expressed as a protein of 393 amino acids, it can also be expressed as smaller products. Independent studies have suggested that *p53* can be alternatively spliced in three different ways (Courtois et al., 2004). The first p53 variant to be discovered lacked the carboxy terminus: its last 26 amino acids were replaced by 17 new amino acids and the alternatively spliced product was named ASp53. It was identified in mouse fibroblasts and has never been shown to be naturally present in humans. Another alternatively spliced p53 protein, I9+, has an alternative carboxy terminus and defective DNA-binding activity. It is present in most normal human cells and tissues. A third alternatively spliced p53 isoform affects the amino-terminus of p53 and is detectable in both normal and transformed cells. The presence of an internal initiation ATG translation site has also been brought to light recently. A p53 fragment lacking its first 39 amino acids (Δ Np53) has been detected in most human cell lines and

tissues (Courtois et al., 2004). The function of these p53 variants resulting from alternative splicing and alternative translation initiation is still poorly understood.

1.4.2. p53 as a transcription activator

p53 can integrate a range of different signals such as DNA damage and oncogenic stress and decide which is the appropriate response. It is upstream of a number of different pathways including DNA repair, cell cycle arrest and apoptosis and it is able to coordinate the necessary response for a particular stimulus. The main mechanism for p53 to activate all these downstream pathways is by acting as a transcription factor and inducing the expression of various genes which will ultimately lead to a suitable response to the stimulus. Indeed, the transcriptional activation function of p53 is a major component of its biological effects (Crook et al., 1994; Pieterse et al., 1994). p53 target genes have been identified by various different approaches such as differential display, cDNA microarray analysis and direct cloning of the p53-binding sequences from human genomic DNA.

Mdm2, the negative regulator of p53

The proto-oncogene Mdm2 was identified as a gene induced by wild-type p53 in an assay using a temperature-sensitive p53 mutant (Barak et al., 1993). Murine *Mdm2* has two different promoters, one present upstream of the gene known as P1, and the other situated near the 3' end of the first intron, known as P2 (Barak et al., 1994). Transcripts from both promoters possess similar coding potentials since the translation start site is located within exon 3. Only the second, P2 promoter is activated by p53, resulting in a mRNA with a transcription site that starts at exon 2 (Barak et al., 1994). The upstream promoter is constitutively active and is only slightly affected by p53. The Mdm2 protein can bind to p53 and inhibit its activity, suggesting a negative feedback loop between p53 and Mdm2 as described in more detail in section 1.4.5 (Marmè et al., 1992) (figure 1.4).

Apoptosis

Following DNA damage, oncogenic activation or withdrawal of growth factors, p53 can induce apoptosis. The first evidence that p53 could induce apoptosis came from the study which showed that p53 reintroduction in a *p53*-deficient myeloid leukaemic cell line induced apoptosis (Yonish-Rouach et al., 1991). In accordance with this observation, normal thymocytes were seen to undergo apoptosis following DNA damage whereas those from *p53*-null mice did not (Clarke et al., 1993; Lowe et al., 1993). The importance of apoptosis in the tumour suppressor function of p53 was highlighted when it was shown that loss of apoptosis correlated with tumour progression in *p53*-null mice (Symonds et al., 1994). Apoptosis has since been shown to be important in suppressing tumour growth and transformation by oncogenes (Lowe et al., 1994; Symonds et al., 1994).

Knock-in mice expressing transcriptionally dead, but DNA-binding proficient, p53 are defective in apoptosis, suggesting that transactivation activity of p53 is essential for it to promote apoptosis in normal cells (Jimenez et al., 2000). Indeed, p53 has been shown to directly stimulate apoptosis by transactivating genes which act as effectors in the apoptotic pathway. Both of the major apoptotic pathways are stimulated by p53: the mitochondrial pathway and the death receptor pathway.

p53 can stimulate the mitochondrial pathway by transactivating various genes which are part of the Bcl-2 family. *Bax*, *Bid*, *Noxa* and *PUMA* are all pro-apoptotic members of the Bcl2 family and are direct targets of p53 (Miyashita et al., 1994b; Nakano and Vousden, 2001; Oda et al., 2000a; Sax et al., 2002; Yu et al., 2001) (figure 1.4). All those genes have p53-binding elements through which p53 has been shown to

transactivate these genes. These four p53 target genes contain BH3 domains and can bind to the anti-apoptotic members of the Bcl-2 family. An increase in the ratio of pro-apoptotic Bcl2 members to anti-apoptotic Bcl-2 family members results in the release of cytochrome C which ultimately leads to cell death (as described in section 1.3.3). Once cytochrome C has been released from the mitochondria, it must bind Apaf-1 in order to activate caspase 9 which is situated at the top of the caspase cascade. *Apaf-1* has been identified as a target gene of both E2F1 and p53, and can sensitize cells to apoptosis (Moroni et al., 2001).

The discovery of *PIG3* and *FDXR* as p53 target genes strongly suggested that p53 could respond to reactive oxygen species (ROS) by generating oxidative stress in the mitochondria (Hwang et al., 2001; Polyak et al., 1997). Following oxidative stress, the mitochondrial components are degraded, culminating in cell death. Although *PIG3* has a consensus p53 binding element in its promoter, p53 was found to bind the *PIG3* promoter through an alternative pentanucleotide microsatellite sequence (Contente et al., 2002). The number of repeats is polymorphic and is directly correlated with the ability of p53 to bind to the *PIG3* promoter *in vivo*. Interestingly, the proline-rich domain of p53 has been shown to be necessary for *PIG3* transactivation: deletion of the proline-rich region prevents the transactivation of *PIG3* whilst not affecting the transactivation of *p21*, *Mdm2* and *Bax* (Venot et al., 1998). A tumour-derived p53 mutant was found to mimic the loss of the proline-rich domain and, as with the deletion mutant, specifically failed to induce *PIG3* (Roth et al., 2000). Both the deletion mutant and the tumour-derived mutant were defective in inducing apoptosis, suggesting that *PIG3* plays a crucial role in p53-dependent apoptosis (Roth et al., 2000).

As well as targeting the mitochondrial-mediated apoptotic pathway, p53 can also activate the death-receptor apoptotic pathway. In normal cells there is a set ratio of death receptors and what are known as decoy receptors (section 1.3.2.). By inducing the expression of the death receptors Fas/APO1 and DR5/KILLER, p53 shifts the balance towards death-receptor-induced apoptosis (Owen-Schaub et al., 1995; Wu et al., 1997). p53 was also found to transactivate a novel gene, known as *PIDD*, which contains a death domain, suggesting a role in the death-receptor-mediated apoptotic pathway. Overexpression of PIDD results in an increase in apoptosis (Lin et al., 2000).

Two other genes downstream of p53 were found to be involved in the apoptotic pathway. *p53AIP* (p53-regulated apoptosis-inducing protein 1) is induced by p53 through its p53 binding sequence and once expressed is localized at the mitochondria (Oda et al., 2000b). Following DNA damage, p53AIP is expressed in a p53-dependent manner. Induction of p53AIP by human p53 requires a phosphorylation site on the serine 46 of p53; phosphorylation of p53 at this site is correlated with its ability to induce apoptosis. Thus p53AIP is likely to play an important role in apoptosis and p53 phosphorylation at serine 46 regulates the transcriptional levels of *p53AIP*. Another p53-inducible gene is *p53DINP1*. This target protein is present in a complex that can phosphorylate p53 at serine 46, thereby increasing its transactivation of p53AIP1 (figure 1.4). Thus, p53DINP1 acts in a positive feedback loop with p53, ultimately inducing p53-mediated apoptosis.

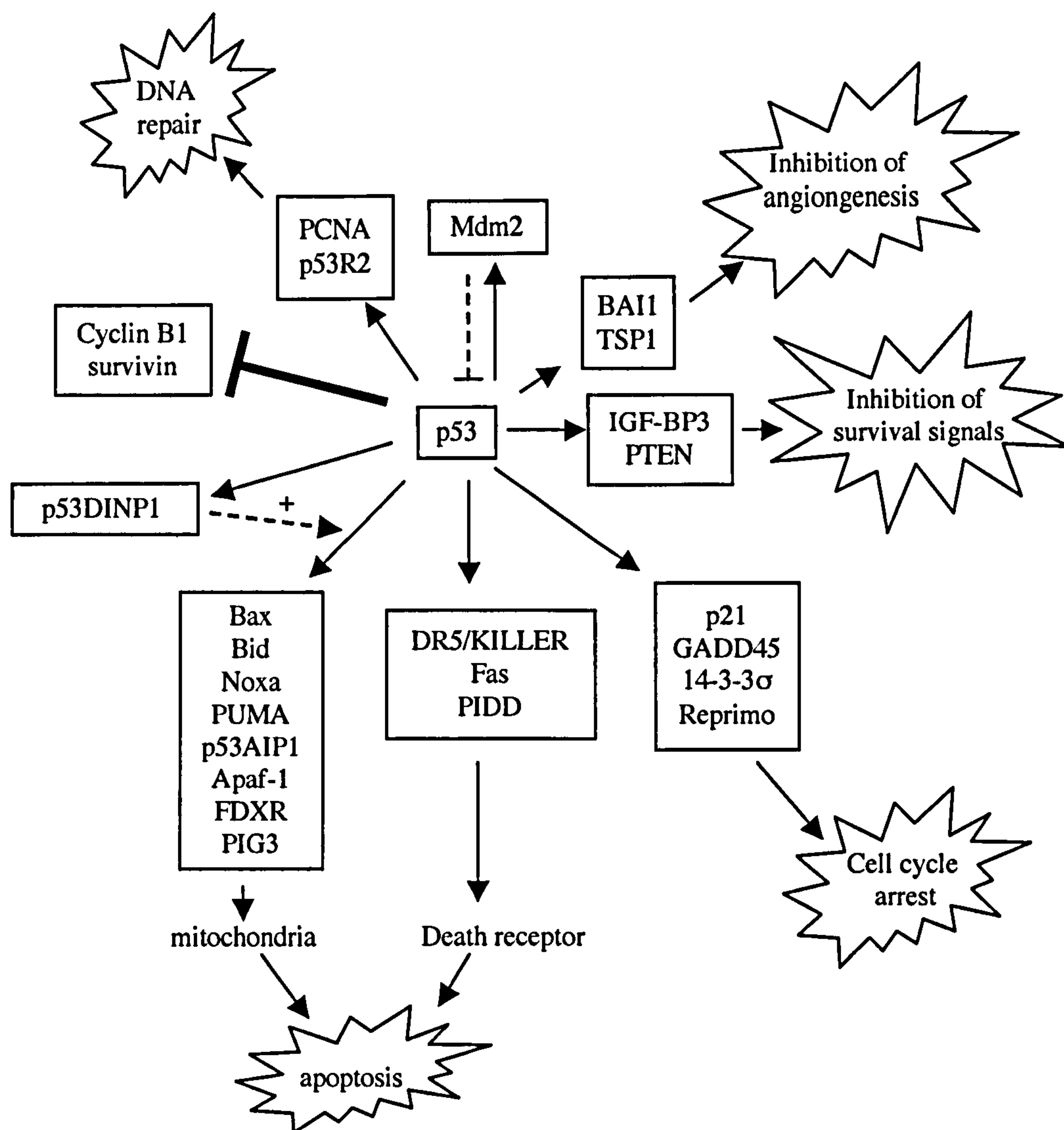


Figure 1.4 *p53* target genes. p53 is upstream of several different effectors. It transcriptionally activates most genes (continuous arrows) but can also repress genes (represented by the bold perpendicular line). Some of its target genes can regulate p53 activity (dotted lines), for example Mdm2 inhibits p53 activity, whereas p53DINP1 induces p53 transcription of the pro-apoptotic genes such as p53 AIP1. p53 can induce apoptosis via both the death receptor pathway and the mitochondria-mediated pathway. p53 can also induce cell cycle arrest and DNA repair as well as inhibiting survival signals and angiogenesis.

The fact that p53 can simultaneously target multiple apoptotic pathways to coordinate cell death might explain why no single p53 effector molecule can account for all p53 pro-apoptotic activity. For example, whereas *Bax* disruption compromises p53-mediated apoptosis in oncogenically transformed fibroblasts (McCurrach et al., 1997), it has no obvious effect of p53-mediated apoptosis in normal thymocytes (Knudson et al., 1995). Similarly, disruption of Apaf-1 expression attenuates p53-mediated apoptosis in transformed fibroblasts (Soengas et al., 1999) and melanoma cells (Soengas et al., 2001) but it has no effect on thymocytes in response to ionizing radiation (Marsden et al., 2002). This discrepancy in sensitivity to particular components of the apoptotic pathway demonstrates differences in cell types.

Cell cycle arrest

Following DNA damage or oncogenic stress, p53 can induce cell cycle arrest. There are two main checkpoints during the cell cycle where p53 can arrest the cycling state of the cell: the G1/S boundary and the G2/M boundary (see section 1.2.2).

p21 was discovered simultaneously as a p53 target gene and as an inhibitor of the cell cycle (El-Deiry et al., 1993; Harper et al., 1993). Following induction by p53, it can form part of a quaternary complex with cyclins, cyclin-dependent kinases (Cdks) and the proliferating cell nuclear antigen (PCNA). As part of this complex, p21 can inhibit Cdk function, leading to hypophosphorylated Rb accumulation and resulting in G1 arrest (see section 1.2.1). Following ionizing radiation, the p53-dependent G1 arrest is at least partly due to p21 activity (el-Deiry et al., 1994). *p21*-null fibroblasts are defective in cell cycle arrest in response to irradiation (Brugarolas et al., 1995; Deng et

al., 1995). p21 can also directly inhibit PCNA function in replication, allowing a time lag for the cells to repair any DNA damage before entering the S phase (Flores-Rozas et al., 1994; Waga et al., 1994).

p53 has been suggested to be involved in a mitotic checkpoint because of the observation that whilst *p53* wild-type cells arrest in G2 following treatment with mitotic spindle inhibitors, *p53*-null cells do not arrest and the cells continue to cycle, resulting in aneuploidy (Cross et al., 1995). In accordance with this observation, three p53 target genes are involved in a G2/M checkpoint arrest: *GADD45*, *reprim*o and *14-3-3 σ* (Hermeking et al., 1997; Kastan et al., 1992b; Ohki et al., 2000). Both GADD45 and 14-3-3 σ can inhibit cyclinB/Cdk2, thereby preventing entry into mitosis (Chan et al., 1999; el-Deiry, 1998). As well as preventing entry into mitosis, GADD45 can also block the cell cycle at the G1/S boundary. It interacts with the replication and repair factor PCNA, inhibiting the cell's entry into S phase (Smith et al., 1994).

DNA repair

The p53 target gene *p53R2* contains high similarity to the ribonucleotide reductase small subunit. Inhibition of p53R2 expression reduced ribonucleotide reductase activity, DNA repair and cell survival after genotoxic stress (Tanaka et al., 2000). *p53R2*-null cells have reduced deoxynucleotides triphosphate (dNTP) pools and increased apoptosis (Yamaguchi et al., 2001), suggesting a role for the p53-induced p53R2 in DNA repair. Further evidence that p53 is involved in repair came with the discovery that p53 binds to several proteins involved in DNA repair, such as RP-A, TFIIH and CSB, and that p53 can recognize both irradiated DNA and mismatched DNA through its carboxy-terminal

region. *PCNA*, which is involved in DNA repair and replication, is also a gene target for p53 transcription activation (Shivakumar et al., 1995) and further implicates p53 in the DNA repair process.

Inhibition of survival signals

The insulin-like growth factor binding protein 3 (IGF-BP3) can be induced by p53 following DNA damage (Buckbinder et al., 1995). IGF-BP3 inhibits signalling by insulin-like growth factor, thereby having an antimitogenic role and leading to the suppression of growth. Another p53 target that prevents anti-apoptotic signals is *PTEN* (Stambolic et al., 2001). PTEN is a lipid phosphatase that can attenuate the survival signals from the Akt pathway by dephosphorylating the Akt products, phosphatidyl inositol 3-phosphates.

Inhibition of angiogenesis

p53 can stimulate both thrombospondin-1 (TSP1) and BAI1 expression which are both potent inhibitors of angiogenesis (Dameron et al., 1994; Nishimori et al., 1997). Both these proteins contain the thrombospondin type 1 repeats.

1.4.3. p53 as a transcription repressor

As well as acting as a transcription activator of genes, p53 is also capable of transrepressing genes. p53 can inhibit transcription of genes lacking a p53-binding site, such as *c-fos*, *c-jun*, *IL-6*, *Rb* and *Bcl2* (Donehower and Bradley, 1993; Jackson et al., 1993; Miyashita et al., 1994a). Only genes with promoters containing TATA boxes, not those containing initiator elements, are inhibited by p53 (Mack et al., 1993), suggesting that p53 might sequester TBP. The carboxy terminal region of p53 contains a repression domain that can also interact with TBP (Horikoshi et al., 1995; Shaulian et al., 1995; Subler et al., 1994). As mentioned above, a p53 mutant with residues 22 and 23 altered no longer binds TAFs but still interacts with TBP. These mutants are unable to repress transcription, suggesting that TAF-binding is required for p53-mediated repression.

Apoptosis and cell cycle arrest may be mediated in part by transcriptional repression since E1B (19 KDa), WT1 and Bcl2, oncogenes that block p53 repression activity whilst having no effect on its transactivation activity, can block p53 apoptotic activity (Crook et al., 1994; Maheswaran et al., 1995; Sabbatini et al., 1995a; Shen and Shenk, 1994). Some p53 mutants that are defective in their transactivation activity retain their suppression of oncogenic transformation function and the G1/S checkpoint arrest function (Sal et al., 1995; Unger et al., 1993). p53 can interact simultaneously with Mdm2 and Rb. In this complex p53 retains its ability to repress genes expression and induce apoptosis despite being defective for the transactivation of its target genes such as *p21* (Hsieh, 1999). These results all suggest a correlation between transcriptional repression and apoptosis.

The identification of the transcription repressor Sin3A as a binding partner to p53 provides a possible explanation for p53 repression activity (Murphy et al., 1999) since Sin3A is complexed with histone deacetylases (HDACs), which are known to silence chromatin structures. Interestingly, Sin3A interacts with p53 in the proline-rich domain of p53, which has been shown to be required for p53-mediated apoptosis and for its transcriptional repression activity (Venot et al., 1998).

p53 as a transcriptional repressor can function through a number of different mechanisms. Firstly, it can act by interfering with the function of DNA-binding of transcriptional activators, as seen with the *AFP* gene (Lee et al., 1999a). Secondly, it can interfere with the basal transcriptional machinery, as is the case with cyclin B repression (Krause et al., 2000). Thirdly it can alter the chromatin structure of its target genes by recruitment of HDACs, as with the *Survivin* target gene. Treatment with trichostatin A (TSA), an inhibitor of HDACs, can abolish p53-mediated repression of survivin (Hoffman et al., 2002; Mirza et al., 2002). *Survivin* encodes an apoptosis inhibitor protein (AIP) that is capable of inhibiting apoptosis (Ambrosini et al., 1997), further emphasizing a link between transrepression and apoptosis.

1.4.4. p53 can induce apoptosis in a transcriptionally-independent manner

A mutant *p53* with mutations in residues 22 and 23 is no longer transcriptionally active nor does it have transrepression activity. This *p53* mutant, however, is nonetheless still able to induce apoptosis in a transient transfection apoptosis assay in HeLa cells (Haupt et al., 1995). In contrast, however, a temperature sensitive form of this same mutant *p53* was not able to induce apoptosis in BKR cells in the presence of E1A (Sabbatini et al., 1995b), highlighting the complexity of the situation. The fact that wild-type *p53* could still induce apoptosis in the presence of either the transcriptional inhibitor actinomycin D or the translational inhibitor cyclohexamide (Caelles et al., 1994; Wagner et al., 1994), is a strong indication that *p53*-mediated apoptosis can occur in a transcription-independent manner.

Recently, it has been shown that *p53* can accumulate in mitochondria following stress, resulting in a cytochrome C-dependent cell death (Marchenko et al., 2000; Mihara et al., 2003). Following the discovery that *p53* can interact with Bcl-X_L and Bcl-2, it has been proposed that *p53* localization to the mitochondria liberates Bax and Bak from the Bcl-2 anti-apoptotic family members, thereby allowing them to induce cytochrome C release from the mitochondria. Interestingly, Bcl2 and Bcl-X_L bind *p53* in its sequence-specific DNA binding region, and most hotspot mutations that disrupt *p53* binding to DNA also disrupt its ability to interact with Bcl-X_L and Bcl-2 (Mihara et al., 2003). In agreement with these studies, Dumont et al. showed that the polymorphic variant of *p53* with an arginine at codon 72 has an increased localization to the mitochondria and this correlates with a greater ability to induce apoptosis (Dumont et al., 2003).

1.4.5. Regulation of p53

Since p53 activation can lead to dramatic responses such as cell cycle arrest or apoptosis, its activity must be tightly regulated. In order for it to function efficiently as a tumour suppressor protein, it must be capable of being activated instantly following the correct stimuli, and then lose its activity rapidly when it is no longer needed. The observation that p53 could still be activated in cells treated with transcription or translation inhibitors suggested that the mechanism for p53 regulation is post-translational (Fritsche et al., 1993). Further work demonstrated that p53 could be regulated at several levels: protein levels, protein-protein interactions, post-translational modification and sub-cellular localization. These different methods of regulating p53 are not discrete units but are interdependent. For example, the protein level of p53 is tightly correlated with its post-translational modifications, and the affinity of the interaction between p53 and its binding partners can be affected by p53 post-translational modifications.

1.4.5.1. Post-translational regulation of p53

There are several types of post-translational modifications that can affect p53 activity: ubiquitination, phosphorylation, acetylation and sumoylation. The use of antibodies to recognize specific covalent modifications such as phosphorylation or acetylation has been a powerful approach to study the effect of post-translational modifications on p53 function.

Phosphorylation

p53 is rapidly activated following DNA damage. This is correlated with the presence of a number of phosphorylation sites altered after DNA damage, leading to a more stable and more active p53. There are two main regions of p53 that are phosphorylated: the amino terminal transactivation domain and the carboxy terminal domain.

Amino terminal region

There are several different kinases that can phosphorylate the amino-terminal region of p53, including DNA PK, ATM, ATR, Chk1 and Chk2. Phosphorylation of p53 by these kinases plays an important role in p53 stabilization and activation by impairing the interaction of p53 and Mdm2

The prevailing view is that DNA damage is sensed by the presence single-stranded DNA (ssDNA) and DNA double stranded breaks (DSBs), although the chromatin conformation has also been implicated in the sensing of DNA damage (Bakkenist and Kastan, 2003; Nyberg et al., 2002; Zhou and Elledge, 2000). ATM and ATR are two members of the PI3K family, located immediately downstream of the damage sensors. ATM is mutated in patients suffering from ataxia telangiectasia (AT) and these patients have a predisposition to cancer among other phenotypes. Both kinases are stimulated following different types of damage: ATM responds to ionizing radiation (IR) whereas ATR responds mainly to ultra-violet (UV) radiation, although it can also be activated somewhat more slowly by IR. Both these kinases phosphorylate and activate the Chk proteins: ATM phosphorylates Chk2 (Matsuoka et al., 1998; Matsuoka et al., 2000; Melchionna et al., 2000) whilst ATR phosphorylates Chk1 (Liu et al., 2000b; Sanchez et al., 1997).

Three p53 phosphorylation sites are directly linked to DNA damage, although other sites can also be phosphorylated in response to stress: serine 15, serine 33 and serine 37, which are all situated in the transactivation domain of p53 (figure 1.5). Different stress signals can give rise to different patterns of phosphorylation, partly due to the fact that distinct stress signals activate specific kinases. Whilst IR stimulation of ATM can phosphorylate p53 at serine 15 (Banin et al., 1998), UV stimulation of DNA PK and ATR can lead to both serines 15 and 17 phosphorylation (Lees-Miller et al., 1992; Tibbetts et al., 1999). The kinetics of p53 phosphorylation following different damages can vary, as seen with serine 15 phosphorylation following UV irradiation that has slower kinetics compared to phosphorylation of the same residue after IR treatment (Shieh et al., 1997; Shieh et al., 1999; Siliciano et al., 1997; Tibbetts et al., 1999). Phosphorylation of p53 on different sites can follow a temporal order, for example ATM and ATR phosphorylation of p53 on serine 15 precedes phosphorylation on serine 20 by Chk2 and possibly Chk1 (Iliakis et al., 2003). In cells from AT patients, lacking a functional ATM, p53 induction in response to IR is significantly delayed, as is its phosphorylation at serine 15 (Kastan et al., 1992a; Khanna and Lavin, 1993). Nonetheless, although delayed, p53 still becomes phosphorylated at serine 15 in AT cells in response to IR, and this is believed to be mediated by ATR (Tibbetts et al., 1999).

As well as inhibiting Mdm2 binding to p53, phosphorylation at the amino terminus domain of p53 can also enhance recruitment of p300 and PCAF to p53, resulting in increased acetylation of p53 and increased stability (see below).

ATM not only phosphorylates p53, but it can also phosphorylate the p53 negative regulator Mdm2. Phosphorylation of Mdm2 by ATM can impair the ability of Mdm2 to target p53 for degradation, further stabilizing p53 (Maya et al., 2001). The kinase JNK can also affect p53 stability following UV damage by phosphorylating p53 on tyrosine 81, leading to p53 stabilization (Buschmann et al., 2001b).

Carboxy-terminal region

The carboxy terminal region of p53 is believed to act as an autoinhibitory domain (see section 1.4.1). Two kinases have been shown to phosphorylate p53 in this region: CKII and PKC. Following DNA damage, some sites in this region are phosphorylated whilst others are dephosphorylated.

CKII phosphorylates p53 at serine 392 (389 in murine p53) and this covalent modification can increase p53 activity (figure 1.5). Indeed, a point mutation at residue 392 from a serine to a glutamate residue rescues p53 function (Hao et al., 1996). One possible explanation for this enhanced activity of p53 following phosphorylation is that TBP or p300, which can both interact with p53 in the carboxy terminal region and are known to be implicated in the repression function of p53, might require phosphorylation at serine 392 to interact with p53. Whereas phosphorylation at serine 392 increases p53 tetramerization, phosphorylation by Cdks at serine 315 in the linker region reverses tetramerization. Phosphorylation of p53 at serine 315 by S and G2/M Cdks can also stimulate p53 DNA binding to a subset of p53 sites (figure 1.5) (Wang and Prives, 1995).

In unstressed cells, PKC phosphorylation of p53 at serine 376 enhances ubiquitination and degradation of p53 (figure 1.5) (Chernov et al., 2001). Following IR, p53 loses this PKC phosphorylation site and the ensuing dephosphorylation is correlated with p53 increased ability to bind to 14-3-3, leading to the stimulation of its site-specific DNA binding function and transactivation activity (Waterman, 1998).

Acetylation

Following phosphorylation of p53 at serines 15 and 33, p300/CBP can acetylate p53 lysines at residues 373 and 382 (figure 1.5) (Gu and Roeder, 1997). Another acetyl transferase that can covalently modify p53, following p53 phosphorylation in the amino terminus region, is PCAF, by acetylating lysine 320 (Sakaguchi et al., 1998). Acetylation at lysines 320, 373 and 382 is modified in response to UV and IR damage (Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998). Although lysine 320 is acetylated following both UV and IR-induced stress, it is an earlier event following UV treatment compared to IR (Sakaguchi et al., 1998). After acetylation, p53 has an increased DNA binding activity (Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998), and acetylation at residues 320 and 373 can increase the expression of *PIG3* and *Noxa* genes, resulting in increased apoptosis (Terui et al., 2003). This suggests that stabilization of p53 by Mdm2 dissociation is not the only mechanism to induce p53 in response to DNA damage.

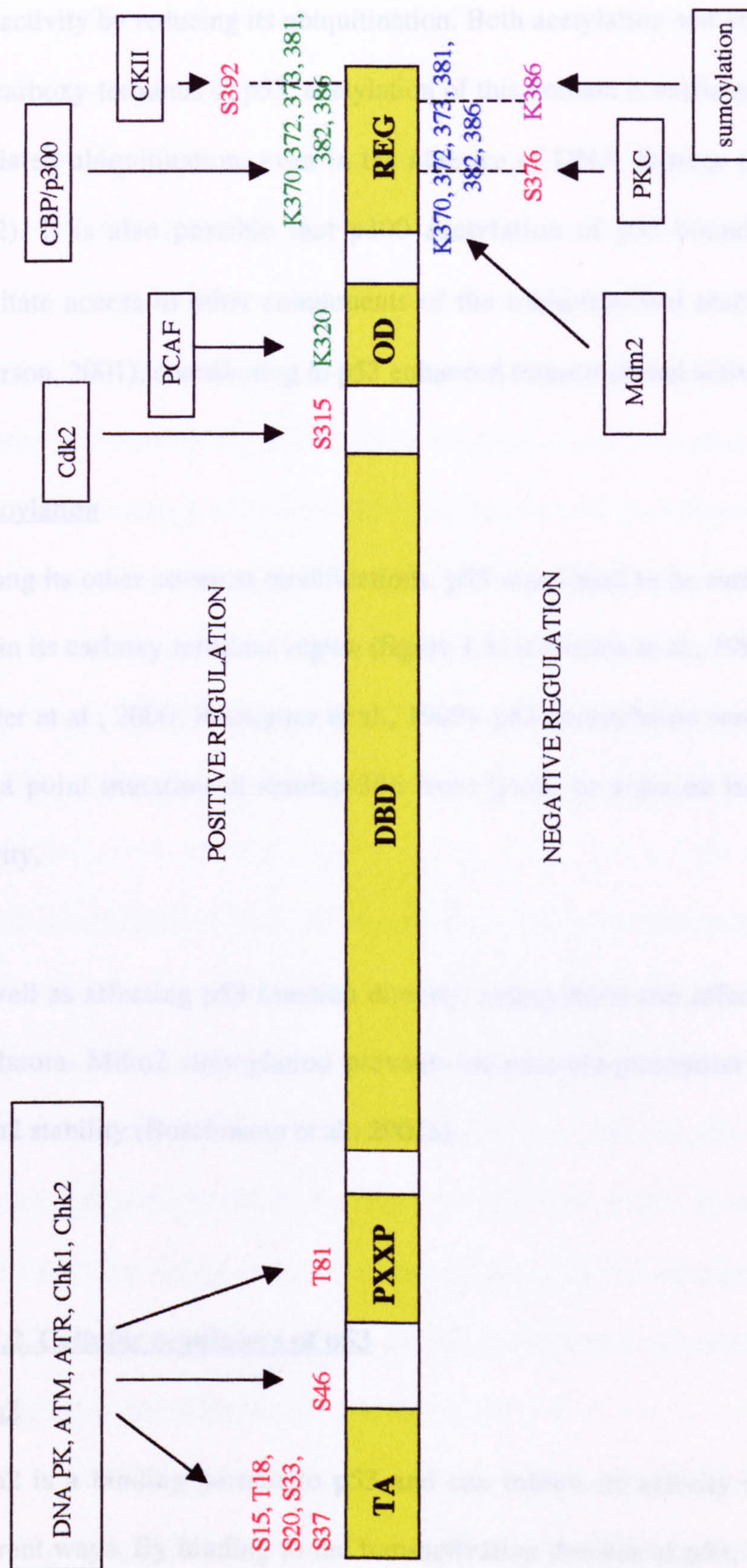


Figure 1.5. *Post-translational modifications of human p53.* The transactivation domain (TA), proline rich domain (PXXP), DNA binding domain (DBD), oligomerization domain (OD) and regulatory carboxy terminal domain (RD) are indicated. Above the p53 protein the positive regulatory post-translational modifications are shown and below the p53 protein are the negative regulatory post-translational modifications. Residues that are phosphorylated are shown in red, those that are acetylated are shown in green, those that are sumoylated are shown in magenta and those that are ubiquitinated are shown in blue. Some of the proteins that mediate the post-translational modifications are indicated.

As well as increasing the DNA binding domain activity of p53, acetylation can regulate p53 activity by reducing its ubiquitination. Both acetylation and ubiquitination occurs in the carboxy-terminus of p53; acetylation of this domain is sufficient to abrogate Mdm2-mediated ubiquitination, even in the absence of DNA damage (figure 1.5) (Li et al., 2002). It is also possible that p300 acetylation of p53-bound nucleosomes could facilitate access to other components of the transcriptional machinery (Espinosa and Emerson, 2001), contributing to p53 enhanced transcriptional activation.

Sumoylation

Among its other covalent modifications, p53 was found to be sumoylated at lysine 386 within its carboxy-terminus region (figure 1.5) (Gostissa et al., 1999; Kwek et al., 2001; Muller et al., 2000; Rodriguez et al., 1999). p53 sumoylation results in p53 repression and a point mutation at residue 386 from lysine to arginine leads to increased p53 activity.

As well as affecting p53 function directly, sumoylation can affect the function of p53 regulators. Mdm2 sumoylation prevents its auto-ubiquitination leading to increased Mdm2 stability (Buschmann et al., 2001a).

1.4.5.2. Cellular regulators of p53

Mdm2

Mdm2 is a binding partner to p53 and can inhibit its activity through a number of different ways. By binding to the transactivation domain of p53, Mdm2 can conceal it from basic transcriptional machinery and coactivators (Momand et al., 1992). Mdm2

has also been shown to recruit the transcriptional repressor CtBP2 to p53, further inhibiting its transactivation activity (Mirnezami et al., 2003). The realization that Mdm2 can negatively regulate p53 by affecting its stability stemmed from the observation that overexpression of Mdm2 causes degradation of p53 and that mutations in *p53* or *Mdm2* that prevent interaction between the two proteins leads to p53 stabilization (Haupt et al., 1997; Kubbutat et al., 1997). Since Mdm2 is an E3 ubiquitin ligase for p53 (Honda et al., 1997), it was suggested that Mdm2 might negatively regulate p53 stability by adding ubiquitin molecules to the carboxy-terminal region of p53, thereby tagging p53 for the 26S proteasome-mediated degradation (figure 1.5). In accordance with this model, Mdm2-mediated degradation of p53 is blocked by the presence of proteasome inhibitors (Haupt et al., 1997; Kubbutat et al., 1997). Unlike many E3 ubiquitin ligases, Mdm2 can only mono-ubiquitinate p53, raising the possibility that another factor is needed for polyubiquitination (Lai et al., 2001).

Mdm2 can further regulate p53 by shuttling p53 from the nucleus to the cytoplasm where it is no longer able to act as a transcription activator or repressor (Roth et al., 1998). Once in the cytoplasm, p53 is available to the proteasome complex and p53 degradation ensues, although it has also been shown that nuclear export is not required for proteasomal degradation of p53 (Xirodimas et al., 2001). It has been suggested that Mdm2 requires cofactors to fully ubiquitinate p53. In accordance with this theory, Mdm2 has been found to bind p300 and this interaction is required for Mdm-2 mediated degradation of p53 (Grossman et al., 1998).

Mdm2 is a transcriptional target of p53, thereby forming a negative feedback loop for p53 activity (Barak et al., 1993; Perry et al., 1993; Wu et al., 1993). This feedback loop

was elegantly demonstrated using knock-out mice: loss of *Mdm2* induces early embryonic lethality that is entirely dependent on p53 function, since the double knock-out of both *p53* and *Mdm2* rescues the phenotype (Jones et al., 1995; Montes de Oca Luna et al., 1995).

As much as p53 activity must be curbed during normal cell growth, p53 must be functionally active following DNA damage and other stimuli. In these circumstances, Mdm2 is no longer an effective negative regulator of p53 and p53 levels are increased as a consequence. Mdm2 interaction with p53 can be blocked by two main ways: covalent modification of p53 or Arf activation. Both of these mechanisms will be discussed below.

MdmX

MdmX is a Mdm2 homolog and can also regulate p53 activity (Shvarts et al., 1996). However, unlike Mdm2, Mdmx does not promote p53 ubiquitination and therefore does not target p53 for degradation but was instead found to increase p53 stability (Jackson and Berberich, 2000; Stad et al., 2000). This increase in p53 stability is thought to occur by Mdmx preventing p53 nuclear export (Stad et al., 2001). Nonetheless, Mdmx can block p53 activity by inhibiting its transcriptional activation of target genes, thereby acting as a negative regulator of p53 (Jackson and Berberich, 2000; Stad et al., 2000). Paradoxically, as well as increasing p53 stability, Mdmx can also increase Mdm2 expression levels by preventing Mdm2 autobiquitination (Stad, 2001). The importance of MdmX as a negative regulator of p53 activity was highlighted by the embryo lethality phenotype of the *MdmX* knock-out mice that was rescued by simultaneous loss of p53 (Parant et al., 2001).

Arf

Contrary to their proliferative effects, some oncogenes, such as Ras, myc and E2F1, can cause p53 accumulation. This stimulation of p53 is thought to be at least partially mediated by Arf: Ras, myc and E2F1 can all induce Arf (Bates et al., 1998; Palmero et al., 1998; Zindy et al., 1998), and overexpression of Arf leads to growth arrest and repression of transformation by Ras in *p53* wild-type but not in *p53*-null cells (Kamijo et al., 1997; Pomerantz et al., 1998). Oncogenic stimulation of Arf by myc and E2F1 is mediated by DAP kinase (Raveh et al., 2001).

Arf stimulates p53 transcriptional activity by binding to the p53 negative regulator Mdm2 and preventing Mdm2-mediated degradation of p53 (Kamijo et al., 1997; Pomerantz et al., 1998; Stott et al., 1998; Zhang et al., 1998). Arf inhibits Mdm2 activity by sequestering Mdm2 in the nucleolus, preventing its nuclear-cytoplasmic shuttling and inhibiting its ubiquitination of p53 (Honda and Yasuda, 1999; Tao and Levine, 1999; Weber et al., 1999). However, Mdm2 nucleolar relocation is not essential for Arf to stabilize p53 (Llanos et al., 2001). Arf can bind directly to both p53 and Mdm2 and the three proteins can be found in a ternary complex (Kamijo et al., 1998). Its role as a tumour suppressor was confirmed in *Arf* knock-out mice, where accelerated tumour development was observed compared to wild-type mice (Sherr and Weber, 2000). p53 and Arf act in a negative feedback loop, since p53 is able to downregulate Arf expression (Stott et al., 1998).

The Arf checkpoint is genetically different in regulating p53 from the DNA damage checkpoint, as *Arf*-null cells have intact DNA damage checkpoints (Kamijo et al., 1997)

and E1A can induce p53 without p53 phosphorylation at serine 15. Thus p53 can be stimulated by DNA damage and by oncogenic stress through two independent pathways.

E2F1

E2F1 was first considered to be an oncogene as it drove the proliferating cell into S phase following release from Rb (Dyson, 1998). Studies in knock-out mice, however, suggested that E2F1 could also act as a tumour suppressor: *E2F1*-null mice were predisposed to tumour development (Dyson, 1998). In accordance with its role as a tumour suppressor, E2F1 was found to induce apoptosis, in both a p53-dependent and p53-independent manner. The mechanism by which E2F1 induces p53-dependent apoptosis is not yet clear but is believed to involve the cyclin A binding site of E2F1: p53 and cyclin A compete in their interaction with E2F1 (Hsieh et al., 2002). Following UV treatment of cells, there is an increased association of E2F1 and p53 that is correlated with decreased cyclin A levels and increased apoptosis (Hsieh et al., 2002).

Thus E2F1 can activate p53 by two independent mechanisms: by inducing Arf, leading to an inhibition of Mdm2-mediated degradation of p53, and by interacting with p53 directly.

Viral proteins

p53 was originally discovered as a binding partner to the SV40 large T antigen (Lane and Crawford, 1979a; Linzer et al., 1979), following which the tumour suppressor gene was found to interact with a number of viral oncogenes. Adenovirus E1B, Human

Papilloma virus (HPV) E6 and the SV40 large T antigen all associate with and inhibit the transactivation activity of p53 (Mietz et al., 1992; Moran, 1993).

PML

Promyelocytic leukaemia (PML) protein is present within the nucleus in specialized structures known as nuclear bodies (NBs). PML can directly interact with the core domain of p53 to recruit p53 to the NBs. Once in the NBs, p53 forms a complex with PML and p300/CBP. This complex enhances p53 transactivation activity (Fogal et al., 2000) and PML is required for p53 to induce apoptosis and senescence.

HIPK2

Also present in the nuclear bodies with p53 and PML is HIPK2, a kinase that can phosphorylate p53 on serine 46. Phosphorylation on this site is important for cell death and leads to increased p53-dependent transactivation of p53AIP1 (Oda et al., 2000b). As well as inducing apoptosis, phosphorylation at serine 46 can, when in concert with acetylation at lysine 382, lead to cell cycle arrest (Hofmann et al., 2002). Since both HIPK2 and p300/CBP are present in NBs, one can suppose that this dual-modification is not uncommon. The overall response to serine 46 phosphorylation, whether cell cycle arrest or apoptosis, might depend in part on other post-translational modifications of p53.

JMY

JMY can interact with p300 and is involved in regulating p53-dependent apoptosis (Shikama et al., 1999). JMY, p300 and p53 form a ternary complex that can increase p53 transactivation activity. JMY is one of the first regulators of p53 found to

specifically promote one p53-dependent response rather than another (in this case, apoptosis).

c-Abl

c-Abl interacts with p53 through its SH3 domain with the p53 proline-rich domain and enhances p53 activity (Goga et al., 1995). It can activate p53 by inhibiting Mdm2-mediated p53 ubiquitination and nuclear export (Sionov et al., 2001). c-Abl contributes to the G1 arrest checkpoint following IR stimulus in a p53-dependent and p21-independent manner (Yuan et al., 1996).

53BP1

53BP1 was found to bind to wild-type p53 but not to mutant p53 in a yeast two hybrid screen (Iwabuchi et al., 1994). It contains BRCT domains with high homology to the Rad9 DNA damage checkpoint protein. Following IR stimulation, 53BP1 changes its subcellular localization from a diffuse nuclear staining to nuclear foci, representing sites of double stranded breaks (Schultz et al., 2000). 53BP1 is required for a subset of ATM-dependent phosphorylation events and for cell cycle arrest at the G2/M boundary of the cell cycle following exposure to IR (DiTullio et al., 2002).

The ASPP family

The carboxy terminus of ASPP2 was found to bind wild-type p53 but not mutant p53 in a yeast two hybrid screen (Iwabuchi et al., 1994). It was originally named 53BP2, until the full length protein was cloned, at which point it was renamed ASPP2 for apoptosis stimulating protein of p53. ASPP1 has high sequence similarity with ASPP2 and both proteins bind p53 in its core domain and enhance its transactivation activity to

specifically target pro-apoptotic genes only (Samuels-Lev et al., 2001). The third member of the family is iASPP, which is an inhibitory protein and prevents p53-mediated apoptosis (Bergamaschi et al., 2003b). More detail on these proteins is described in section 1.6.

1.4.5.3. p53 regulation by subcellular localization

As mentioned above, p53 can shuttle in and out of the cytoplasm and nucleus. The nuclear localization signals are present in the carboxy-terminus of p53, whereas the two nuclear export signals are at a distance from each other; one is in the oligomerization domain and the other is in the transactivation domain.

The intrinsic ability of p53 to be exported from the nucleus is enhanced in the presence of Mdm2 (Freedman et al., 1999) and is dependent on the ubiquitin ligase function of Mdm2 (Boyd et al., 2000; Geyer et al., 2000). One possible explanation is that the nuclear export signal situated in the carboxy terminus of p53 is unmasked following ubiquitination. By a different mechanism, the nuclear export signal situated in the transactivation domain is regulated by phosphorylation: following stress, this site is phosphorylated, thereby inhibiting this export signal (Zhang and Xiong, 2001). Although nuclear export is not necessary for p53 degradation (Xirodimas et al., 2001), p53 is nonetheless degraded more effectively in the cytoplasm (Freedman and Levine, 1998).

Although nuclear export of p53 is usually seen as a way to inhibit its activity, the recent discovery that p53 can localize to the mitochondria and thereby induce apoptosis

(Marchenko et al., 2000; Mihara et al., 2003), could suggest that p53 nuclear export might not necessarily be a negative regulation of p53.

Within the nucleus, p53 is found in different discrete structures, including nucleoli and nuclear bodies (NBs). Nucleolar p53 is associated mainly with mdm2 and Arf whereas p53 found in the NBs is associated with PML and p300.

negative regulators of p53	p53 activating proteins
<p>By degradation: Mdm2 <i>E6 (HVP)</i></p> <p>By phosphorylation: PKC</p> <p>By other means: MdmX iASPP <i>large T antigen (SV40)</i> <i>E1B (adenovirus)</i></p>	<p>By phosphorylation: DNA PK JNK ATM ATR Chk1 Chk2 CKII HIPK2</p> <p>By acetylation: p300/CBP PCAF JMY</p> <p>By other means: Arf E2F1 PML TBP TAFs c-Abl 53BP2 ASPP1 ASPP2/53BP2</p>

Table 1.2 *Summary of p53 binding proteins.* Viral proteins are shown in italics with the virus species in brackets. All other proteins are cellular proteins.

1.4.6. Choice of response: cell cycle arrest or apoptosis

In response to many different stimuli, p53 can stimulate a variety of responses. The most prominent responses that the tumour suppressor induces are cell cycle arrest and apoptosis. How one or the other of these two responses becomes favoured is not yet fully understood but the factors involved are slowly coming to light.

A number of factors can affect the outcome of p53 response, such as the presence of overexpressed viral or cellular oncoproteins, growth factor availability and expression of Rb/E2F1. Loss of Rb function caused by viral proteins correlates with a loss of G1 arrest following DNA damage (Demers et al., 1994; Hickman et al., 1994; Slebos et al., 1994). This relationship between p53 and Rb explains why several DNA tumour viruses inactivate both tumour suppressors (e.g. adenovirus, HPV).

Two possible models have been proposed to explain p53 selectivity for either cell cycle arrest or apoptosis (Vousden and Lu, 2002).

p53 “dumb” model

In this model, p53 always sends exactly the same signals following activation, and induces both cell cycle arrest and apoptosis target genes. Inhibition of p53-induced apoptosis by extrinsic components reveals the parallel p53-dependent cell cycle arrest, and vice versa.

Some cell types, such as thymocytes, show a propensity to undergo apoptosis following p53 activation, whereas others tend to arrest. This difference might be due to the transcription factors present in the different cell types. The fact that cell cycle arrest

genes and apoptotic genes are differentially regulated within the same cell type can be explained by the availability of different transcription factors that bind the promoters of these genes.

In addition, there is an enhanced sensitivity of transformed cells to undergo apoptosis in response to p53 activation, which explains why p53 activation preferentially kills tumour cells while sparing normal tissue. E2F1, for example, although an oncogene, can synergize with p53 to induce apoptosis (Hsieh et al., 1997b). Not all oncogenes can preferentially select for apoptosis, however; NF- κ B, for example, is known to inhibit cell death. At high levels, NF- κ B can compete with p53 for the p300 coactivator, blocking p53 apoptotic activity (Ravi et al., 1998a; Webster and Perkins, 1999). Myc overexpression can also influence p53 response: p53 no longer induces p21 expression because Myc and its binding partner Miz1 are present at the *p21* promoter, preventing p53-mediated transcription (Fridman and Lowe, 2003). Therefore, in the presence of myc overexpression, p53 induces apoptosis following activation. In these cases, the presence of a particular oncogene in the cell would determine the p53 response rather than the intrinsic activity of p53 itself.

Some oncogenes do not generate straight-forward activation or inhibition of p53 responses. Ras, for example, can stimulate p53 activity by inducing Arf expression. Following Arf expression, p53 relocalizes to the nucleoli, and induces senescence (Weber et al., 1999). Parallel to Arf activation, Ras can also stimulate Akt which is upstream of survival signals, inhibiting p53-mediated responses (Sabbatini and McCormick, 1999). In the case of Ras, the final response of cells to these stimuli will be the result of the integration of a complex network of signals.

p53 “smart” model

In this model, p53 itself governs the choice of response appropriate for the stimuli. It would be expected that different forms of p53 could affect the choice. For example, it is known that low amounts of p53 can induce cell cycle arrest whereas higher levels leads to apoptosis. One possible explanation for this observation is that p53 binds the pro-apoptotic promoters with a lower affinity than to the cell cycle arrest promoters and the pro-apoptotic promoters would therefore only be activated following higher p53 protein levels.

Another example when p53 could modulate its own activity is seen with its phosphorylation state at serine 46. Phosphorylation at serine 46 is required to induce the expression of the apoptotic gene *p53AIP1* (Oda et al., 2000b), resulting in a apoptotic response.

IR and UV radiation can induce different p53 target genes in the same cell types (Zhao et al., 2000). These distinct stimuli lead to different post-translational modifications, which can influence p53 in its choice of targets. Similarly, DNA damage and hypoxia produce different p53 modifications that correlate with the ability of p53 to associate with different transcriptional coactivators and repressors (Koumenis et al., 2001).

Some co-factors of p53 are known to direct p53 to stimulate one particular response over another. ASPP1, ASPP2 and JMY can target p53 to induce activation of apoptotic genes specifically (Samuels-Lev et al., 2001; Shikama et al., 1999) (see section 1.6). The modulation of the availability of these co-factors or the regulation of their ability to

interact with p53 could be key in determining whether a cell undergoes apoptosis or cell cycle arrest in response to p53 activation. In accordance with this theory, ASPP inhibition can block p53-mediated apoptosis (Samuels-Lev et al., 2001). In addition to co-factors, p53 requires at least one member of its family, p63 or p73, for an apoptotic response (Flores et al., 2002).

Further in agreement with this model, it is known that cell cycle arrest and apoptosis inductions are separate functions of p53. Some tumour-derived p53 mutants have defects in activation of pro-apoptotic promoters but not cell cycle arrest targets (Friedlander et al., 1996; Ludwig et al., 1996), suggesting an intrinsic ability of p53 to decide which response to activate.

1.4.7. *p53* and tumourigenesis

More than 50% of cancers have missense mutations in the *p53* gene, of which 28% of mutations affect only six residues: 175, 245, 248, 249, 273, 282 (Hollstein et al., 1994). These mutations are selected to prevent p53 binding to DNA in a sequence-specific manner. The importance of p53 as a tumour suppressor is indisputable; patients with the Li-Fraumeni syndrome have germline mutations of *p53* and are predisposed to cancer. Similarly, *p53* knock-out mice are also predisposed to tumour development (Donehower et al., 1992). Following the two-hit system for tumour suppressors described by Knudson (Knudson, 1971), it is typical to find one *p53* allele deleted whilst the prevailing one is mutated. Confirming this model, the majority of heterozygous *p53* mice developing tumours have the remaining *p53* allele mutated in the tumours. It is not necessary for both *p53* alleles to be dysfunctional or deleted, however, for p53 to have reduced activity. *p53* heterozygous cells show dosage effects in chemically induced mouse skin tumours, suggesting that the level of p53 can influence the phenotype (Kemp et al., 1993).

The impact of altered or loss of p53 function might be greater than originally thought: tumours with wild-type *p53* often have mutations in the upstream regulators or downstream effectors of p53, preventing p53 normal function. For example, *Mdm2* is amplified in one third of all sarcomas, and these tumours all have wild-type *p53* (Oliner et al., 1992). Similarly, *ASPP1* and *ASPP2*, the p53 co-factors that can induce p53-dependent apoptosis, have reduced expression in breast carcinomas, whereas *iASPP*, the family member that inhibits p53-dependent apoptosis, is overexpressed in a number of tumours (Bergamaschi et al., 2003b; Samuels-Lev et al., 2001). Thus, in cases where *p53* is wild-type, loss of function of p53 regulators can lead to p53 dysfunction.

The temporal occurrence of p53 mutations varies in tumours; p53 mutations can occur late in some cancers, such as in colorectal cancers, and early in some pre-malignant lesions, such as in skin cancers. The selection for p53 inactivation could be caused by a variety of factors such as tissue environment and oncogenic overexpression. An understanding of the tumour cell environment selecting for *p53* inactivation came with the observation that p53 can be induced by hypoxic conditions, such as happens when tumours have inadequate blood supplies (Graeber et al., 1994). Therefore cells lacking functional p53 have a growth advantage in hypoxic conditions.

Most mutant p53 proteins are more stable than their wild-type counterpart and are present at higher levels. Some mutants are thought to act as dominant negative inhibitors of wild-type p53, although this activity is generally not sufficient since many tumours that harbour point mutations also show loss of heterozygosity, thereby eliminating the wild-type allele. However, it has been shown in some cases of mice with a single dominant negative p53 mutant, that this is sufficient to develop tumours without the loss of wild-type *p53* (Liu et al., 2000a). Some p53 mutants, such as p53 R175H, provide gain of function phenotypes (Gualberto et al., 1998). These mutants can provide a selective survival advantage following chemotherapy treatment, possibly compromising the efficiency of chemotherapy (Blandino et al., 1999). The gain of function of some p53 mutants might be due to their ability to interact with other p53 family members.

As mentioned above (sections 1.4.3 and 1.4.4), p53 does not always require its transactivation activity to induce apoptosis. However, the selection for p53 mutants that

have defective DNA binding activity (known as hotspot mutations) strongly suggests that transcription activation is critical for p53 to function as a tumour suppressor. Some tumour-derived p53 mutants can activate *p21* but not *Bax* (Friedlander et al., 1996), suggesting that cell cycle arrest induction is not sufficient for p53 to function as a tumour suppressor. In agreement with these observations, studies have linked *p53* loss to apoptotic defects during the progression of murine and human tumours, emphasizing the role of apoptosis in p53 tumour suppressor function (Attardi and Jacks, 1999; Bardeesy et al., 1995).

p53 polymorphism

A single nucleotide polymorphism is found at codon 72 of p53, within its proline-rich domain. This codon can either encode an arginine (R) or a proline (P) residue. The p53 polymorphism can affect the risk of developing some tumours (Buller et al., 1997; Wang-Gohrke et al., 1998).

p53 polymorphism can also influence tumour response to drug therapy: the wild-type p53 72R polymorphism has an increased ability to induce apoptosis following chemotherapy, compared to wild-type p53 72P (Dumont et al., 2003; Sullivan et al., 2004). This difference is at least in part mediated by the greater ability of wild-type p53 72R to localize to the mitochondria (Dumont et al., 2003). Interestingly, a p53 mutant with the 72R codon has decreased sensitivity to chemotherapy drugs compared to a p53 mutant with 72P codon, in head and neck cancers (Bergamaschi et al., 2003a). The ability of mutant p53 72R to provide survival signals to cells following drug treatment might be due to its interaction with other p53 family members: mutant p53 72R can bind to p73 and inhibit its pro-apoptotic activity with a higher affinity than the mutant

p53 72P (Marin et al., 2000). The arginine polymorphic form of p53 is preferentially mutated in squamous cell tumours arising in polymorphic heterozygotes, suggesting that inactivation of the p53 family members may contribute to the oncogenic properties of a subset of p53 mutants (Marin et al., 2000).

1.5. The p53 family members: p63 and p73

Twenty years after the discovery of the tumour suppressor protein p53 (Lane and Crawford, 1979a; Linzer and Levine, 1979), two other members of the family were identified: p63 and p73 (Kaghad et al., 1997; Osada et al., 1998; Schmale and Bamberger, 1997; Trink et al., 1998; Yang et al., 1998; Zeng et al., 2001). All three family members are structurally related and share homology in three domains: the transactivation domain, the DNA-binding domain and the oligomerization domain. As well as these three domains, p63 and p73 also contain an additional domain in their carboxy-terminus, the SAM motif. Although the SAM motif found in other proteins is known to be involved in protein-protein interactions, its role in p63 and p73 has not yet been uncovered.

p63 and p73 share a high level of homology with p53 in all three common domains, although the DNA binding domain shows a particularly striking homology with 60% identity in all three proteins. The DNA-binding domains of p63 and p73 have an even higher level of identity between themselves at 86% amino acid identity and 91% amino acid homology. All the p53 residues that contact DNA or are involved in the structural scaffolding of the p53 DNA binding domain, known as hotspots due to their frequent mutation rates, are conserved in all three family members.

Unlike p53, both p63 and p73 have structurally complex genes. Both genes have several carboxy-terminus alternative splice variants, giving rise to many different proteins. In addition, p63 and p73 have two alternative promoters: P1 is situated in the 5' untranslated region (UTR) and is upstream of the non-coding exon 1, whereas P2 is present within intron 3. All transcripts that start from the P1 promoter include a

transactivation (TA) domain in their amino terminus, whilst all the transcripts starting from the P2 promoter lack the TA domain and are known as ΔN .

TAp63 and TAp73 have a similar function to p53. They can transactivate many p53-target genes, such as *p21*, *14-3-3 σ* , *GADD45*, *PIGs*, *p53R2*, *Mdm2* and *Bax*, and can also induce apoptosis (Nakano et al., 2000; Steegenga et al., 1999; Zhu et al., 1998). As with p53, p73 can also repress transcription (Salimath et al., 2000). On the other hand, the ΔN isoforms of p63 and p73 act as dominant negative inhibitors of their full-length isoforms and of p53.

p53 does not play an important role in development but is essential for preventing tumour formation: *p53* knockout mice are viable but are predisposed to tumours (Donehower et al., 1992). Unexpectedly, although p63 and p73 have similar transcription and apoptotic activities as p53, their roles differ significantly. Knockout mice studies show that neither p63 nor p73 prevent the formation of tumours although they both are important players in mouse development: *p63* knockout mice have epidermal developmental defects (Mills et al., 1999; Yang et al., 1999a) whilst *p73* knockout mice have considerable neuronal defects (Pozniak et al., 2002; Yang et al., 2000). Confirming the importance of p63 as an important player in development, it was found that heterozygous germline mutations of *p63* in humans cause the autosomal dominant developmental disorder EEC (Ectrodactyly, Ectodermal Dysplasia, Facial Clefts). The p63 mutations from these patients reside within its DNA-binding region and prevent TAp63 transcription activation whilst $\Delta Np63$ cannot act as a dominant negative protein in these patients.

1.5.1. Are p63 and p73 tumour suppressor proteins?

The *p73* gene frequently undergoes loss of heterozygosity (LOH) in some cancers, suggesting that it can act as a tumour suppressor (Kaghad et al., 1997). However, unlike the classic tumour suppressor protein described by Knudson (Knudson, 1971), it is rare to find *p73* loss of function mutations. Additionally, p73 fails to be inactivated by viral oncoproteins that can inactivate p53, such as SV40 T antigen and the adenovirus E1B 55 KDa protein, further suggesting that p73 is not a tumour suppressor.

Unlike tumour suppressor proteins, p73 is overexpressed in a majority of cancers (Kovalev et al., 1998; Zaika et al., 1999). Paradoxically, p73 is methylated in many tumour types, such as lymphomas, gliomas and pancreatic carcinomas, consistent with tumour suppression function (Corn et al., 1999; House et al., 2003; Martinez-Delgado et al., 2002; Siu et al., 2002; Watanabe et al., 2002). The levels of p73 in tumours can be correlated with the prognosis: patients with higher levels of p73 expression have a worse survival rate than patients with undetectable levels of p73 (Tannapfel et al., 1999).

Whereas TAp63 isoforms do not seem to vary in tumours compared to normal tissue, the Δ Np63 dominant negative isoforms are often overexpressed in tumours, due to the amplification of the gene.

1.5.2. Regulation of p63 and p73

As with p53, both p63 and p73 can be stabilized in the presence of proteasome inhibitors. However, unlike p53, the proteasome-mediated degradation of p73 is not mediated by Mdm2 (Gu et al., 2000). It therefore comes as no surprise that the tumour suppressor Arf, that can increase p53 levels by inhibiting Mdm2, has no effect on p73 stability (see section 1.4.5). Nonetheless, Mdm2 can still bind to p73 and inhibit its transcription activity; the p73-Mdm2 complex prevents p73 binding its co-factor p300/CBP, thereby reducing p73 activity (Zeng et al., 1999). As with p53, Mdm2 is a gene target for p73 transactivation, and therefore acts as a negative feedback loop (Balint et al., 1999; Dobbelstein et al., 1999; Zeng et al., 1999). p73 is also resistant to other oncogenes that can mediate p53 degradation, such as the viral protein HPV E6.

Whilst TAp63 levels are dependent on proteasome-mediated degradation, its $\Delta Np63$ isoforms can be regulated independently of proteasomes. The regulation of the dominant negative form of p63 has actually been shown to be dependent on p53, further connecting the family members together.

As seen in section 1.4.5, p53 activity can be regulated by post-translational modifications, occurring mostly in response to DNA damage. p73 can also be stabilized and activated in response to some stimuli, such as cisplatin, doxorubicin, taxol and ionizing radiation (IR), but is insensitive to other DNA damage agents such as ultra-violet radiation (UV), actinomycin D and mitomycin C (Bergamaschi et al., 2003a; Fang et al., 1999a; Kaghad et al., 1997). IR induces c-Abl to phosphorylate p73 at tyrosine 99, following a direct interaction between the proline rich region of p73 and the

SH3 domain of c-Abl (Agami et al., 1999; Yuan et al., 1999). The phosphorylated form of p73 has an increased stability (Gong et al., 1999).

p73 can also interact with cyclin-dependent kinases (Cdks) and can be phosphorylated by them on threonine 83 *in vitro*. *In vivo* phosphorylation of p73 at residue 83 is cell-cycle dependent and results in the repression of p73 transactivation activity (Gaiddon et al., 2003).

Another post-translational modification that can affect p73 activity is sumoylation; p73 is sumoylated at its carboxy-terminus on lysine 627, promoting its degradation (Minty et al., 2000). p73 can also be acetylated at residues 321, 327 and 331 in a DNA-damage-dependent manner; these acetylation modifications regulate p73 promoter specificity (Costanzo et al., 2002).

Following high levels of E2F1, Myc or E1A, endogenous TAp73 can be induced, resulting in increased transcriptional activity, apoptosis and growth suppression (Lissy et al., 2000; Stiewe and Putzer, 2000; Zaika et al., 2001). E2F1 can directly regulate TAp73 expression by binding to its P1 promoter and transactivating the gene (Irwin et al., 2000; Stiewe and Putzer, 2000). Since E2F1 is commonly deregulated in many tumours, this might provide an explanation for why p73 is overexpressed in tumours.

As with p53, both p63 and p73 bind to co-factors that regulate their function. ASPP1 and ASPP2 bind to all three p53 family members and specifically enhance their pro-apoptotic activity (Bergamaschi et al., 2004) (see section 1.6). The Wilm's tumour 1

protein (WT1) on the other hand, can inhibit p53 and p73 apoptotic activity (Scharnhorst et al., 2000).

1.5.3. Crosstalk between p53-family members

Although no physical interaction has been shown between p53 wild-type and its family members, some p53 mutants can associate with p73 or p63 (Di Como et al., 1999; Gaiddon et al., 2001; Marin et al., 2000). Additionally, some p53 mutants with the arginine polymorphism at codon 72 can bind p73 with a higher affinity than those with the proline polymorphism, and therefore have a greater ability to inhibit p73 function (Bergamaschi et al., 2003a; Marin et al., 2000) (see section 1.4.7). This interaction might provide one possible explanation for the p53 gain-of-function mutants: by interacting with p73 and inhibiting its pro-apoptotic function, mutant p53 can act as an oncogene.

Wild-type p53, p63 and p73 proteins can also communicate with each other. In an elegant study by Flores et al., it was shown that mouse embryo fibroblasts (MEFs) transformed with E1A, lacking either *p63* or *p73*, show an intermediate resistance to apoptosis in response to doxorubicin treatment, when compared to *p53*-null and wild-type MEFs transformed with E1A (Flores et al., 2002). Surprisingly, *p63* and *p73* double knockout cells (*p63*^{-/-}; *p73*^{-/-}) were as resistant to apoptosis as the *p53* knockout MEFs. This suggests that p63 and p73 are required for p53-induced apoptosis. Pursuing this issue further, it was discovered that although *p63*^{-/-}; *p73*^{-/-} had no effect on p53 transcription activity of endogenous *p21* and *Mdm2* genes, there was a significant reduction in p53 transactivation of the pro-apoptotic genes *bax* and *PERP* (p53

apoptosis effector related to PMP-22). Chromatin-immunoprecipitation (ChIP) assays demonstrated that p63 and p73 are required for p53 binding to its pro-apoptotic target gene promoters specifically, and not to its other target genes such as *p21* and *Mdm2* (Flores et al., 2002). Thus, p53, p63 and p73 cooperate to ensure proper transactivation of pro-apoptotic genes ultimately leading to apoptosis.

As mentioned earlier, the *p73* gene has two different promoters, giving rise to two isoforms. Interestingly, it was discovered that the full length TAp73 can stimulate the expression of the Δ Np73. Adding complexity to the system, p53 was also found to transactivate Δ Np73 from the P2 promoter. Since Δ Np73 can act in a dominant negative fashion to TAp73 and to p53, both cases represent a negative feedback loop, similar to that seen with p53 and Mdm2 (Grob et al., 2001). In addition, Δ Np63 is transcriptionally repressed by p53, further showing cross-talk between the family members (Waltermann et al., 2003).

1.6. The ASPP family

1.6.1. *ASPP1* and *ASPP2*

The first member of the ASPP family to be discovered was found to specifically bind wild-type and not mutant p53 in a yeast two-hybrid screen (Iwabuchi et al., 1994). The protein was named 53BP2 for p53 binding protein 2 and was a partial clone of what is now known as full length ASPP2, spanning residues 528-1128. Unlike other previously described p53-binding proteins, 53BP2 was found to bind p53 in its DNA-binding domain (Gorina and Pavletich, 1996; Iwabuchi et al., 1994). 53BP2 binds p53 through its ankyrin repeat domain and its SH3 domain, situated in the carboxy-terminus of 53BP2 (Gorina and Pavletich, 1996; Iwabuchi et al., 1994).

Understanding the function of the ASPP family was impeded by the fact that, until recently, only partial cDNA clones of *ASPP2* were available. Following the initial discovery of 53BP2 (residues 528-1128 of full length ASPP2), a larger, but still incomplete, clone of *ASPP2* was discovered as a Bcl-2 binding protein and was therefore called Bbp2, for Bcl-2 binding protein 2 (Naumovski and Cleary, 1996). This larger version of 53BP2 is a protein of 1005 amino acids, corresponding to residues 123-1128 of full length ASPP2 (figure 1.6A).

The different sized fragments seem to function in opposing manner: whereas 53BP2 (528-1128 ASPP2) inhibits p53 activity by preventing p53 binding to DNA (Iwabuchi et al., 1994), the larger clone Bbp2 (123-1128 ASPP2) stimulates p53 activity by increasing p53 transactivation (Iwabuchi et al., 1998). Nonetheless, there remains some controversy about the role Bbp2 plays in regulating p53 function: whilst some studies

demonstrated that Bbp2 stimulates p53 pro-apoptotic function (Ao et al., 2001; Yang et al., 1999b), others showed that Bbp2 impedes cell cycle progression at G2/M in a p53-dependent manner (Naumovski and Cleary, 1996). Bbp2 (123-1128 ASPP2) was also shown to partially suppress E1A and Ras-mediated transformation of rat embryo fibroblasts (REFs) and to inhibit clonogenic survival of the HEK293 cell line (Ao et al., 2001; Iwabuchi et al., 1998). Taken together, the above observations strongly indicate that Bbp2 (residues 123-1128 of ASPP2) enhances p53 tumour suppressor activity.

The discovery of the full length ASPP2 and its close homolog, ASPP1, helped clarify some of the misunderstandings (Samuels-Lev et al., 2001). Both ASPP1 and ASPP2 are large proteins, containing 1190 residues and 1128 residues, respectively. Their highest homology regions are situated in their amino termini, which contain a putative alpha helix domain, and their carboxy termini, which contain ankyrin repeats and an SH3 domain (Figure 1.6A). ASPP1 and ASPP2 were so called to highlight their structure (ankyrin repeat, SH3 domain and proline-rich domain-containing proteins) and their function (apoptosis stimulating protein of p53). All the ASPP2 residues that contact p53 are conserved in ASPP1 and the two proteins have a similar function in stimulating p53 activity.

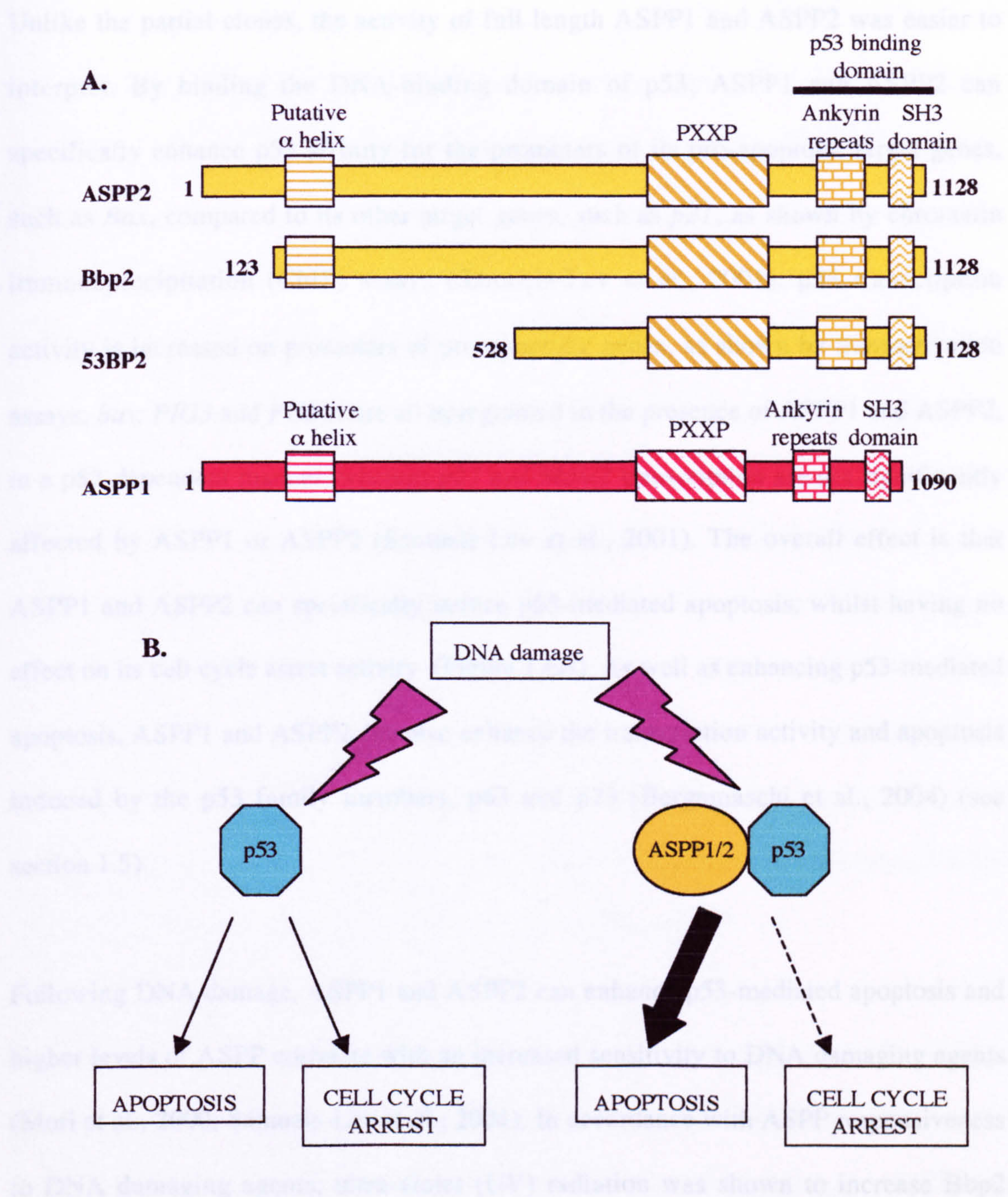


Figure 1.6. *ASPP1 and ASPP2 structure and function.* (A) Schematic diagram of ASPP1 full length (1-1090), ASPP2 full length (1-1128) and its partial clones Bbp2 (123-1128) and 53BP2 (528-1128). All four proteins contain a proline-rich region (ASPP1: 669-860; ASPP2: 696-882), ankyrin repeats (ASPP1: 920-982; ASPP2: 958-1023) and an SH3 domain (ASPP1: 1021-1076; ASPP2: 1060-1116). ASPP1, ASPP2 and Bbp2 also contain a putative α helix domain in their amino terminus. Both ASPP1 and ASPP2 have a putative nuclear export signal (ASPP1: 267-271; ASPP2: 234-246) and a putative nuclear localization signal as described by Sachdev (1998) (ASPP1: 889-894; ASPP2: 927-932). (B) p53 can induce either apoptosis or cell cycle arrest in response to DNA damage. ASPP1 and ASPP2 can specifically enhance p53-dependent apoptosis activity without inducing cell cycle arrest.

Unlike the partial clones, the activity of full length ASPP1 and ASPP2 was easier to interpret. By binding the DNA-binding domain of p53, ASPP1 and ASPP2 can specifically enhance p53 affinity for the promoters of its pro-apoptotic target genes, such as *Bax*, compared to its other target genes, such as *p21*, as shown by chromatin immunoprecipitation (ChIP) assays (Samuels-Lev et al., 2001). p53 transcription activity is increased on promoters of pro-apoptotic genes, as shown by transactivation assays: *bax*, *PIG3* and *PUMA* are all upregulated in the presence of ASPP1 and ASPP2, in a p53-dependent manner, whereas *p21* and *Mdm2* transcription are not significantly affected by ASPP1 or ASPP2 (Samuels-Lev et al., 2001). The overall effect is that ASPP1 and ASPP2 can specifically induce p53-mediated apoptosis, whilst having no effect on its cell cycle arrest activity (Figure 1.6B). As well as enhancing p53-mediated apoptosis, ASPP1 and ASPP2 can also enhance the transcription activity and apoptosis induced by the p53 family members, p63 and p73 (Bergamaschi et al., 2004) (see section 1.5).

Following DNA damage, ASPP1 and ASPP2 can enhance p53-mediated apoptosis and higher levels of ASPP correlate with an increased sensitivity to DNA damaging agents (Mori et al., 2000; Samuels-Lev et al., 2001). In accordance with ASPP responsiveness to DNA damaging agents, ultra-violet (UV) radiation was shown to increase Bbp2 (residues 123-1128 of ASPP2) levels in a p53-independent manner (Lopez et al., 2000). Although ASPP2 is predominantly found in the cytoplasm, it has occasionally been seen in the nucleus (Samuels-Lev et al., 2001); the ankyrin repeat of ASPP2 has been found to contain a novel class of nuclear import signal, suggesting a putative role in the nucleus (Sachdev et al., 1998).

The crystal structure of the p53-53BP2 complex revealed that the p53 residues that contact 53BP2 are evolutionarily conserved. Furthermore, the p53 residues that interact with 53BP2 are commonly mutated in tumours. In light of ASPP1 and ASPP2 function, the relevance of the p53 mutants seems more pertinent than ever. p53 mutants that cannot be stimulated by ASPP1 and ASPP2 will no longer be able to induce apoptosis effectively. It was thus not unexpected to discover that several tumour-derived p53 mutants are defective for ASPP2 binding without having lost their ability to bind DNA, such as those with mutations in residues H178, H179, M243, N247 and R282 (Gorina and Pavletich, 1996; Thukral et al., 1994). Additionally, there exists a tumour-derived p53 mutant with a mutation at residue 181 that, although able to bind ASPP2, can no longer be regulated by ASPP2. Moreover, the implication that ASPP2 could be necessary for p53 tumour suppressor function came with the observation that the p53 residues that contact both 53BP2 and DNA (R248, R273) were the residues most commonly mutated in human tumours, compared to the residues that contact either DNA or 53BP2 (Cho et al., 1994; Gorina and Pavletich, 1996).

If ASPP1 and ASPP2 regulation is necessary for p53 tumour suppressor function, as these p53 mutants suggest, it is expected that tumours that have retained wild-type p53 will no longer have functional ASPP1 and ASPP2 proteins. Indeed, in a screen of breast carcinomas containing wild-type p53, it was shown that 60% of tumours had reduced expression of ASPP1 and 23% had a decrease in ASPP2 levels (Samuels-Lev et al., 2001). In addition, ASPP2 has been mapped to chromosome 1q41-42 (Iwabuchi et al., 1998), a site that has been found to be deleted in cervical carcinomas (Sreekantaiah et al., 1988).

Since ASPP1 and ASPP2 can induce p53-mediated apoptosis, it is expected that they are stringently regulated. How these proteins are regulated is not yet known, although it is likely to involve interaction with their binding partners. Although ASPP1 has so far not been much studied, ASPP2 truncation mutants (53BP2 and Bbp2) interact with many different proteins, mainly through the use of the yeast-two hybrid system. It is worth taking note that none of the interactions discovered by the yeast-two hybrid system between the partial ASPP2 clone and its binding partners were confirmed by co-immunoprecipitations.

One of the first proteins found to interact with Bbp2 (residues 123-1128 of ASPP2) was the anti-apoptotic protein Bcl-2 (see section 1.3.3). Bcl-2 and p53 are unable to bind Bbp2 simultaneously and they therefore compete for Bbp2 binding (Naumovski and Cleary, 1996). Although it is clear that ASPP2 can increase p53-mediated apoptosis by increasing its binding to promoters of pro-apoptotic genes, it is also possible that ASPP2 may induce apoptosis by inhibiting Bcl-2 anti-apoptotic function.

The p65 RelA member of the NF- κ B family, can also bind Bbp2 (residues 123-1128 of ASPP2) in a yeast-two hybrid system (Yang et al., 1999b). The NF- κ B family members are transcription factors that are critical regulators of cellular responses following various stimuli and are involved in both pro-apoptotic and anti-apoptotic pathways. The most common form of NF- κ B is a heterodimer composed of p50 and p65, with the p65 subunit being the active transcriptional component (Nolan et al., 1991). NF- κ B is retained in the cytoplasm by its inhibitory family member, I κ B. Following stimulation, such as the TNF α responsive pathway, I κ B gets phosphorylated and dissociates from NF- κ B, which is then free to translocate to the nucleus and transactivate its many target

genes. Bbp2 interacts with the central region of p65. Although the effect of p65 on ASPP2 is still unclear, p65 can suppress Bbp2-mediated apoptosis (Yang et al., 1999b), suggesting it might act as an inhibitor to ASPP2.

Bbp2 (123-1128 ASPP2) was found to interact with the catalytic subunit of protein phosphatase 1 (PP1) in a yeast-two hybrid screen (Helps et al., 1995). As with Bcl-2, binding of PP1 and p53 to Bbp2 is mutually exclusive. PP1 interacts with a variety of regulatory subunits that modulate its activity. Bbp2 can regulate PP1 activity in some cases only: it abolishes PP1 activity towards glycogen phosphorylase but has no effect on PP1 activity towards myosin P-light chains, suggesting a selective effect on PP1 substrate specificity (Helps et al., 1995). Further work by Susana Llanos in our laboratory confirmed that full length ASPP1 and ASPP2 could bind PP1 *in vivo*, and they did so with a higher affinity than for p53 (Llanos, unpublished).

Another Bbp2 binding partner discovered by the yeast-two hybrid system is the Yes-associated protein (YAP). The WW1 domain of YAP interacts directly with the SH3 domain of Bbp2. Overexpression of c-Yes leads to tyrosine phosphorylation of Bbp2, and this phosphorylation can affect YAP-Bbp2 interactions (Espanel and Sudol, 2001).

Yet another protein known to bind the ASPP2 truncation mutant, Bbp2, is APCL. APCL is highly homologous to the tumour suppressor APC and is expressed mainly in the central nervous system. Co-expression of APCL with Bbp2 causes Bbp2 relocation from the cytoplasm to the perinuclear region (Nakagawa et al., 2000).

1.6.2. iASPP

A third member of the ASPP family was recently discovered and named iASPP for inhibitor of the ASPP proteins. Unlike ASPP1 and ASPP2, that could both enhance p53-pro-apoptotic activity, iASPP specifically inhibits p53 pro-apoptotic activity. iASPP has high homology to ASPP1 and ASPP2 in its carboxy-terminus region, especially the ankyrin repeats, the SH3 domain and the proline-rich region. However, it has no homology to the two pro-apoptotic family members in its amino-terminus region.

iASPP had previously been identified as an inhibitor of the NF- κ B family member, RelA, and has therefore been named RAI for RelA-associated inhibitor. RAI was shown to bind to the p65 subunit and inhibit its transcriptional activity by preventing p65 binding to DNA (Yang et al., 1999b). As a consequence of its structural homology to ASPP1 and ASPP2 and of its role in ASPP-p53 mediated apoptosis, it was renamed iASPP by our laboratory.

iASPP is the most evolutionarily conserved member of the family, present in humans and *Caenorhabditis elegans* (*C. elegans*) (Bergamaschi et al., 2003b). Inhibition of iASPP expression can induce p53-mediated apoptosis in *C. elegans* and human cells, and overexpression of iASPP confers resistance to DNA damage-induced apoptosis. In addition, iASPP can cooperate with Ras, E1A and E7 to transform rat embryo fibroblasts (REFs) (Bergamaschi et al., 2003b). The role of iASPP as an oncoprotein was confirmed when it was found to be overexpressed in breast carcinomas containing wild-type p53 and normal levels of ASPP1 and ASPP2 expression (Bergamaschi et al., 2003b). Taken together, these results indicate that iASPP is a novel oncogene and could

provide an important factor in determining the necessary therapy for a particular tumour.

1.7. Ras

Ras is a key mediator of signal transduction in all eukaryotic cells, from yeast to humans. It acts as a molecular switch by binding to guanine nucleotides with high affinity: when inactive it is bound to GDP, and following stimulation, it is bound to GTP and is “turned on”. Ras senses extracellular signals from growth factors, cytokines, hormones and neurotransmitters and relays them to a number of different cytosolic effector proteins. Following activation, Ras can influence cell growth, differentiation and apoptosis.

1.7.1. Ras: structure-function relationship

Three Ras genes

Humans and rodents encode three functional Ras genes: *H-Ras*, *K-Ras* and *N-Ras*, that are dispersed in different chromosomes (Lowy and Willumsen, 1993). The three *Ras* genes have a common structure with a 5' noncoding exon and four coding exons. The exons are similar for all three genes but the sizes of the introns differ widely between genes, resulting in large differences in the sizes of the *Ras* genes. The three *Ras* genes have promoters with high GC content and no TATA motif, which is characteristic of housekeeping genes. Some of the regions controlling Ras expression are found in the 5' region of the genes and in the first intron (Jeffers and Pellicer, 1994; Paciucci and Pellicer, 1991).

Following alternative splicing of the fourth exon, the *K-Ras* gene encodes two proteins, K-rasA and K-rasB (McGrath et al., 1983). All four Ras proteins are either 188 or 189

amino acids long. Different Ras genes are expressed in different tissues at different levels but at least one Ras protein is found in all tissues (Lowy and Willumsen, 1993). All three Ras genes share 85% amino acid identity. They are highly homologous in their first 164 amino acids, whilst their last 25 amino acids diverge significantly except for a conserved cysteine four residues from the carboxy terminus.

Ras is a guanine nucleotide-binding protein

As mentioned above, Ras can bind both GDP and GTP with high affinity. When bound to GDP, Ras is inactive but following stimulation, Ras binds GTP and is then able to activate its downstream effectors. Ras has an intrinsic GTPase activity that can hydrolyze GTP to GDP, returning Ras to its inactive state (Gibbs et al., 1984; Manne et al., 1985; Sweet et al., 1984). The higher intracellular GTP concentration compared to GDP means that upon dissociation of GDP, Ras will normally bind GTP. Most Ras proteins in normal cells are found in their inactive GDP-bound state. There are four sequence motifs in Ras involved in nucleotide interactions: residues 10-17 (binds the α and β phosphates), residues 57-60 (binds Mg^{2+} and γ phosphate of GTP) and the two regions at 116-119 and 144-147 that both bind the guanine ring (Lowy and Willumsen, 1993).

Ras-GTP has a different conformation than Ras-GDP. These conformational changes described in crystal structures are concentrated in two regions: residues 30-38 (loop2/switch1 region) and residues 60-76 (loop4/switch2 region) (Milburn et al., 1990; Schlichting et al., 1990). Switch I region is the main effector binding site, responsible in part for interactions with GTPase activation proteins (GAPs) and downstream effectors. The Switch II region interacts mainly with guanine nucleotide exchange factors

(GNEFs). Other residues outside of switch I and II regions have also been found to be critical for Ras signalling even though they do not change their conformation upon ligand exchange; these residues are designated “activator region” or “constitutive effector region” (Fujita-Yoshigaki et al., 1995; Marshall, 1993). Therefore, depending on whether Ras is bound to GDP or GTP, it can interact with its regulatory proteins and signal to its downstream effector proteins (Malumbres and Pellicer, 1998).

1.7.2. Regulation of Ras

Following, extracellular stimulation, Ras can be activated by guanine nucleotide exchange factors (GNEFs) that dissociate GDP from Ras, allowing Ras to bind to GTP and thereby become active. GTPase activating proteins (GAPs) negatively regulate Ras. Ras has a very slow intrinsic GTPase activity, and can only efficiently hydrolyze GTP to GDP in the presence of a GAP.

Upstream activators of Ras

Acting as intermediates between extracellular signals and Ras are transmembrane receptors. These receptors interact with the extracellular signalling molecules directly, and consequently activate Ras by recruiting a GNEF. There are different types of receptors that can activate Ras: tyrosine kinase receptors, T cell receptors and subunits of heterotrimeric G proteins.

The most studied route for Ras activation is the epidermal growth factor (EGF) signalling through its receptor, EGFR. Following association of its extracellular segment with EGF, EGFR dimerizes and autophosphorylates itself in its intracellular portion on tyrosine residues. The phospho-tyrosine residues on EGFR are binding sites for SH2 domains and recruit the adaptor protein Grb2 to the cell surface. Grb2 is in a stable association with the GNEF Sos which consequently is also recruited to the plasma membrane (Chardin et al., 1993; Gale et al., 1993; Lowenstein et al., 1992; Rozakis-Adcock et al., 1992). Sos1 and Sos2 are widely expressed GNEFs. They are related to the son-of-sevenless (Sos) *Drosophila* product which functions in eye development (Bonfini et al., 1992; Bowtell et al., 1992). Sos is specific for H-Ras and N-Ras and is unable to catalyze the nucleotide exchange for the Ras-related proteins

RalA or Cdc42. Once in proximity with Ras, Sos can dissociate GDP from Ras. The high GTP/GDP intracellular ratio ensures that the released nucleotide is replaced with GTP. Sos then dissociates, leaving Ras in its active form where it can bind a number of effector proteins and activate various downstream signalling pathways (Bourne et al., 1990; Bourne et al., 1991).

Although Sos is the best studied GNEF, there are others that can also activate Ras. rasGRF is homologous to the *Saccharomyces cerevisiae* (*S. cerevisiae*) Cdc25 (Wei et al., 1992) and is expressed specifically in the brain. It activates H-Ras but not N-Ras nor K-rasB (Jones and Jackson, 1998). rasGRF2 is stimulated not by receptor tyrosine kinases but by calcium influx, following which it translocates from the cytosol to the cell periphery (Fam et al., 1997; Malumbres and Pellicer, 1998).

GAPs

The most well-studied GTPase activating protein (GAP) is p120-GAP. It was isolated from *Xenopus* and found to bind preferentially to Ras-GTP (Vogel et al., 1988). p120-GAP can accelerate the intrinsic GTPase activity of normal Ras by at least five orders of magnitude (Gideon et al., 1992; Trahey and McCormick, 1987). p120-GAP is active on H-Ras, K-Ras and N-Ras but not on other Ras family members such as Rho and Rac proteins (Takai et al., 2001).

NF1 is another regulator of Ras. It is responsible for von Recklinghausen's neurofibromatosis (NF1 disease) which is inherited as an autosomal dominant disorder in patients that have benign and malignant tumours. The mutation in the *NF1* gene represents a null allele, strongly suggesting that NF1 acts as a tumour suppressor

protein. The middle region of the protein has sequence and functional homology to the catalytic domain of p120-GAP. Unlike *p120-GAP*, *NF1* is preferentially expressed in cells from the nervous system (Daston and Ratner, 1992; Golubic et al., 1992).

Post-translational modifications and localization

All three Ras proteins are synthesised in the cytoplasm. Post-translational modifications at the carboxy terminus increase the hydrophobicity of the proteins, resulting in their association with the inner face of the plasma membrane (Grand et al., 1987; Willingham et al., 1980). Ras is thought to associate with specific receptors in caveolae microdomains of the plasma membrane (Mineo et al., 1996; Song et al., 1996). The hypervariable domain of Ras, situated in its last 25 amino acids, is essential for membrane association, and the cysteine positioned at residue 186 is required to initiate post-translational modification (Hancock, 2003; Willumsen et al., 1984). Mutation in the last four residues, the CAAX motif, abolishes plasma-membrane localization and signalling of Ras (Willumsen et al., 1984).

The first modification to occur is farnesylation of the cysteine 186 of the CAAX motif (Hancock et al., 1989; Reiss et al., 1990). Following that, the last three amino acids at the carboxy terminus are proteolytically cleaved (Fujiyama and Tamanoi, 1990; Hancock et al., 1989) and the carboxyl group of cysteine 186 is methylated (Dai et al., 1998; Hancock et al., 1991; Hrycyna et al., 1991). Then H-Ras and N-Ras cysteine residues upstream of the farnesylated cysteine become reversibly palmitoylated in their hypervariable region (Buss and Sefton, 1986; Hancock et al., 1989).

Studies using live-cell imaging, fluorescence resonance energy transfer, fluorescence recovery after photobleaching and electron microscopy have shown that the interaction of Ras with the plasma membrane is highly dynamic and that Ras is also present on endosomes, the endoplasmic reticulum and Golgi apparatus (Hancock, 2003). Ras does not translocate directly to the cell surface but first interacts with intracellular membranes (Apolloni et al., 2000; Choy et al., 1999). Ras first interacts with the endoplasmic reticulum, and this interaction requires the CAAX structure and farnesylation. N-Ras and H-Ras then associate with the Golgi apparatus, following which they are transported by exocytic vesicles. K-Ras translocation to the plasma membrane does not involve the Golgi route.

Farnesylation of the carboxy terminus of Ras appears to be important for the high affinity interaction of Ras with its effector Raf, suggesting that the Ras carboxy terminus region may be involved in effector interaction, as well as the switch I and II regions (Campbell et al., 1998; Hu et al., 1995). Ras does not only signal to its effector protein Raf from the plasma membrane, it can also signal to it from the Golgi and the endoplasmic reticulum membranes (Chiu et al., 2002).

Differences in the three Ras proteins

Although the three Ras proteins, H-Ras, K-Ras and N-Ras all seem to signal to similar effectors, there are nonetheless differences between them. They all have different expression patterns according to the organ in which they are present, throughout development and differentiation (Malumbres and Pellicer, 1998) and they have different affinities for interacting proteins; for example NF1 has a higher affinity for H-Ras than N-Ras and rasGRF activates H-Ras but not N-Ras nor K-Ras (Bollag and McCormick,

1991; Jones and Jackson, 1998). As mentioned above, K-Ras does not get palmitoylated and it is likely that it is present at a different microdomain of the plasma membrane to H-Ras and N-Ras, which might affect its activity.

Highlighting the differences in the three *Ras* genes, are the different phenotypes present in transgenic mice with each of the *Ras* genes knocked-out. *N-Ras* knockouts develop and reproduce normally although they have a defective immune response and T-cell function (de Castro et al., 2003; Umanoff et al., 1995). *H-Ras* knockouts have no obvious phenotype although they develop fewer tumours following treatment with carcinogens compared to wild-type mice (Esteban et al., 2001; Ise et al., 2000). *K-Ras* is the only gene that is required for development as its deletion leads to embryo lethality following fetal liver defects and anaemia (Johnson et al., 1997; Koera et al., 1997). Since mice with a double deletion for *H-Ras* and *N-Ras* can still develop normally, K-Ras is required for normal mouse development, suggesting that K-Ras has functions that are not shared by its other family members (Esteban et al., 2001).

1.7.3. The Raf-MAPK pathway

Raf is an effector of Ras

Following the observation that activated Raf can cause transformed and tumourigenic phenotypes indistinguishable from the activated Ras phenotype, it has been suggested that Raf is a downstream effector of Ras (Bonner et al., 1985; Leever et al., 1994; Stanton et al., 1989; Stokoe et al., 1994). In accordance with this hypothesis, dominant negative forms of Raf-1, MEK and MAPKs have been shown to reduce Ras transformation activity (Campbell et al., 1998).

There are three Raf genes: *Raf-1*, *A-Raf* and *B-Raf*, with *B-Raf* existing in multiple spliced forms. All three proteins have been shown to be activated by Ras.

Raf interaction with Ras

Raf binds to Ras in a GTP-dependent manner both *in vitro* and *in vivo* (Moodie et al., 1993; Van Aelst et al., 1993; Vojtek et al., 1993; Warne et al., 1993). GTP-bound Ras translocates Raf-1 to the plasma membrane where Raf gets activated (Stokoe et al., 1994). Raf-1 binds Ras-GTP through its Ras binding domain (RBD) in its conserved region CR1. This initial interaction allows for a second Ras binding site in Raf-1, known as the cysteine-rich domain (CRD), to contact Ras (figure 1.7) (Brtva et al., 1995; Drugan et al., 1996). The CRD associates with different residues of Ras-GTP from those that bind the RBD (Drugan et al., 1996). In the full-length Raf-1 molecule, the CRD is inaccessible for Ras-GTP but previous binding of the RBD domain to Ras unmask the CRD domain, which is then able to interact with Ras.

A mutant Ras that can bind Raf but is still unable to activate it, suggested that Ras binding to Raf is not sufficient to activate Raf and other factors might be needed (Akasaka et al., 1996). Similarly, Raf binding to Ras *in vitro* is not sufficient to stimulate Raf-1 kinase activity (Traverse et al., 1993; Zhang et al., 1993). Following Ras activation, Raf-1 is hyperphosphorylated (Morrison et al., 1993). Overexpression systems and mutational analysis have shown that Raf-1 phosphorylation at tyrosines 340 and 341 enhances its catalytic activity (Marais et al., 1995). Further work showed that the tyrosine kinases that phosphorylate Raf-1 are members of the Src kinase family (Marais et al., 1995; Park et al., 1996). Other kinases have also been suggested to play a role in Raf activation, such as PAK, PKC and PKA (Chong et al., 2003).

14-3-3 is a specific phosphoserine-binding protein (Muslin et al., 1996) and has been shown to interact with Raf-1 and activate it in a way that does not affect Raf interaction with Ras (Fantl et al., 1994; Freed et al., 1994). 14-3-3 is thought to play two roles: it is required to maintain Raf-1 in its inactive conformation in the cytosol prior to Ras stimulation and, following Ras stimulation, it facilitates Raf-1 activation and stabilizes activated Raf-1 (Michaud et al., 1995; Takai et al., 2001). Other components are known to be involved in Raf activation such as the heat shock proteins, Hsp90 and p50, molecular chaperones and phospholipids, although their roles in Raf activation have not yet been clearly defined (Campbell et al., 1998).

Post-translational modifications of Ras are thought to be important for the activation of, but not the association with, Raf (Lerner et al., 1995; Okada et al., 1996). Therefore, post-translational modification of Ras is necessary for both its localization and its

biological activity. In accordance with this, post-translational modification of K-Ras is required for MAPK activation in a cell-free system (Itoh et al., 1993)

Whereas Ras activates Raf-1 by recruiting it to the membrane, it activates B-Raf by inducing a conformational change of B-Raf (Leevers et al., 1994; Yamamori et al., 1995). It is worth noting that these studies were done with oncogenic H-Ras only, not with any of the other Ras proteins which might have a slightly different method of regulating Raf.

Ras can activate the different Raf proteins to different extents; Raf-1 and A-Raf are weakly activated by oncogenic Ras and strongly activated by Src, whereas B-Raf is strongly activated by Ras and not activated by Scr. Raf1 has two tyrosine phosphorylation sites that are involved in Ras-dependent activation of Raf-1; these two sites are missing in B-Raf, suggesting a different mode of regulation for these proteins (Marais et al., 1997).

Raf-MEK-MAPK phosphorylation cascade

Activated Raf kinase can phosphorylate its substrates MAPK extracellular signal- regulated kinases 1 and 2 (MEK1 and MEK2), thereby activating them (figure 1.7) (Kyriakis et al., 1992). Once phosphorylated, the MEK dual threonine/tyrosine kinase activity is stimulated and it can then phosphorylate its downstream substrates mitogen- activating protein kinases 1 and 2 (MAPK1 and MAPK2) also known as Erk1 and Erk2 (figure 1.7) (Gomez and Cohen, 1991; Kosako et al., 1992; Nakielnny et al., 1992). MAPK phosphorylation promotes its homodimerization and results in the translocation of MAPK to the nucleus where it can directly phosphorylate its substrates

(Khokhlatchev et al., 1998). Unlike Raf and MEKs, MAPK1 and MAPK2 have numerous substrates that are present in both the cytoplasm and the nucleus, such as p90 ribosomal S6 kinase (p90^{RSK}) and the transcription factors TCF/Elk1 and Ets2. These transcription factors are involved in ternary complex formation at the serum response elements (SRE), which regulate the expression of immediate-early genes, such as Fos, eventually leading to cell proliferation (figure 1.7) (Gille et al., 1992). Phosphorylation of p90^{RSK}, also known as Rsk, activates it and stimulates its phosphorylation of the transcription factor CREB, making it transcriptionally active (Xing et al., 1996). Rsk is involved in Raf-mediated survival signals and a dominant negative form of Rsk eliminates survival signals from activated MEK alleles (Bonni et al., 1999; Shimamura et al., 2000). The importance of the Raf-MAPK pathway in Ras signalling is confirmed by the observation that constitutively active MEK can mimic the effects of Ras activation in some cell types (Cowley et al., 1994; Mansour et al., 1994).

The kinase suppressor of Ras (KSR) was discovered as a loss-of-function allele that blocked the MAPK signalling pathway and Ras-induced transformation (Denouel-Galy et al., 1998; Joneson et al., 1998). It can directly interact with MEK1, MEK2 and MAPK proteins (Denouel-Galy et al., 1998; Yu et al., 1998) and it has been proposed that it acts as a scaffold protein that links MEK to its substrates MAPK1/2, thereby facilitating signal transmission between these proteins (figure 1.7) (Therrien et al., 1996; Yu et al., 1998).

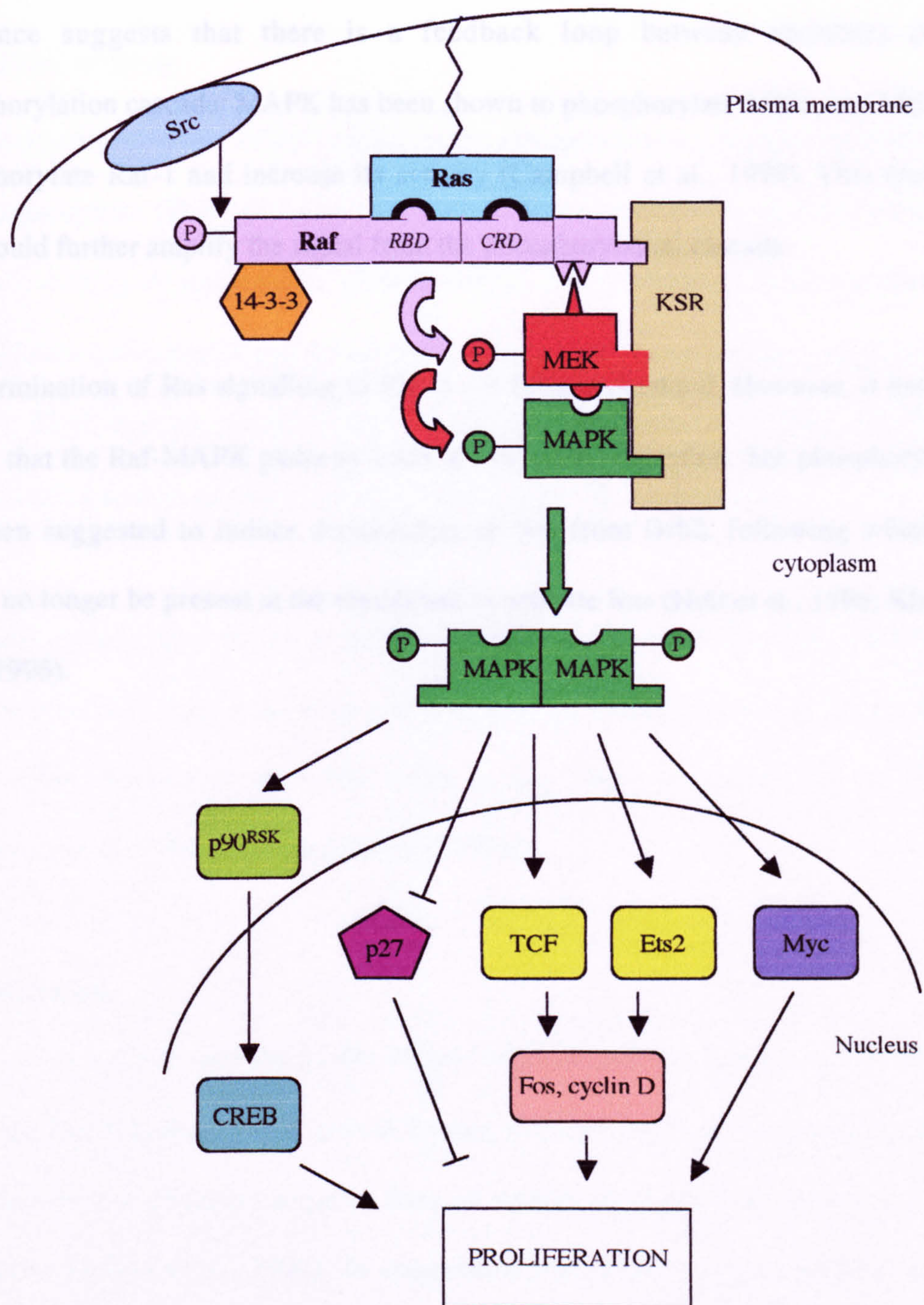


Figure 1.7. *The Ras-Raf-MEK-MAPK pathway.* Following stimulation, Ras recruits Raf to the plasma membrane. Raf binds Ras through two regions, the Ras binding domain (RBD) and the cysteine-rich domain (CRD). Following its recruitment to the membrane, Raf is activated by many factors, including 14-3-3 and Src. Activated Raf phosphorylates MEK which then phosphorylates MAPK. MAPK is then free to phosphorylate various downstream substrates, mostly transcription factors, present both in the cytoplasm and in the nucleus. In response to MAPK, phosphorylated transcription factors transactivate genes involved in proliferation. MAPK can also prevent p27 cell cycle inhibition by targeting it for degradation.

Evidence suggests that there is a feedback loop between members of the phosphorylation cascade: MAPK has been shown to phosphorylate MEK, and MEK can phosphorylate Raf-1 and increase its activity (Campbell et al., 1998). This feedback loop could further amplify the signal from the phosphorylation cascade.

The termination of Ras signalling to Raf is not fully understood. However, it has been shown that the Raf-MAPK pathway leads to Sos phosphorylation. Sos phosphorylation has been suggested to induce dissociation of Sos from Grb2, following which Sos would no longer be present at the membrane to activate Ras (Holt et al., 1996; Klarlund et al., 1996).

1.7.4. PI3K pathway

Phosphoinositol 3 kinase (PI3K) catalyzes the phosphorylation of phosphatidylinositol (4,5)-biphosphate (PtdIns[4,5]P₂) to yield phosphatidylinositol (3,4,5)-triphosphate (PtdIns[3,4,5]P₃) in response to growth factors and cytokines. PI3K are found as heterodimers consisting of a p110 catalytic subunit and a p85 regulatory subunit. There are two PI3K that are activated by receptors tyrosine kinases, PI3K α and PI3K β , corresponding to the catalytic subunits p110 α and p110 β , respectively.

PI3K and its lipid products act on pathways that control cell proliferation, cell survival, membrane ruffling, cell chemotaxis and vesicular trafficking (Fantl et al., 1992; Kotani et al., 1994; Kundra et al., 1994; Schu et al., 1993; Wennstrom et al., 1994a; Wennstrom et al., 1994b; Yao and Cooper, 1995).

PI3K activation

Ras binds to and activates the p110 α subunit of PI3K and can stimulate PI3K activity: following Ras stimulation with growth factors, PtdIns[3,4,5]P₃ levels increase, and this is blocked by the dominant negative form of rasN17 (Rodriguez-Viciana et al., 1996; Rodriguez-Viciana et al., 1994). In accordance with PI3K being an effector of Ras, PI3K is required for some of the effects Ras has on cells (Rodriguez-Viciana et al., 1996; Rodriguez-Viciana et al., 1994; Rodriguez-Viciana et al., 1997). Ras stimulation of PI3K is cooperative with tyrosine phosphopeptide binding to p85 (Rodriguez-Viciana et al., 1996).

PI3K has three main targets: the ribosomal protein p70 S6 kinase (p70^{S6K}) that controls the translation of mRNA transcripts containing polypyrimidine tracts, Rac that is

involved in cytoskeletal organization (Welch et al., 2003), and PKB (also known as Akt) that is involved in cell survival and metabolism (Downward, 1998a), as shown in figure 1.8.

PKB activation

PKB/Akt has a serine/threonine kinase domain and a pleckstrin homology (PH) domain, the latter of which can specifically bind PtdIns[3,4,5]P₃, stimulating PKB translocation to the membrane (Frech et al., 1997; James et al., 1996; Klippel et al., 1997; Stokoe et al., 1997). Once present at the plasma membrane, PKB is phosphorylated on two sites, threonine 308 and serine 473, resulting in its activation (Alessi et al., 1996; Downward, 1998a). Phosphoinositide-dependent protein kinase 1 (PDK1) has been shown to phosphorylate PKB at threonine 308 following PI3K stimulation (figure 1.8) (Alessi et al., 1997; Datta et al., 1999).

Following stimulation, PKB can phosphorylate a number of substrates including glycogen synthase kinase 3 (GSK3) and phosphofructokinase, thereby influencing metabolism (figure 1.8) (Downward, 1998a). PKB can also induce transcription from the Fos promoter and stimulate DNA synthesis in quiescent cells.

PKB is involved in the Ras-mediated survival pathway and protects cells from apoptosis (Berra et al., 1998; Dudek et al., 1997; Kauffmann-Zeh et al., 1997). It can do so by inhibiting the pro-apoptotic proteins Bad and caspase 9, as well as by inhibiting the forkhead transcription factors that are involved in FasL expression (figure 1.8) (see section 1.3.2) (Brunet et al., 1999; Cardone et al., 1998; Datta et al., 1997; Kops et al., 1999).

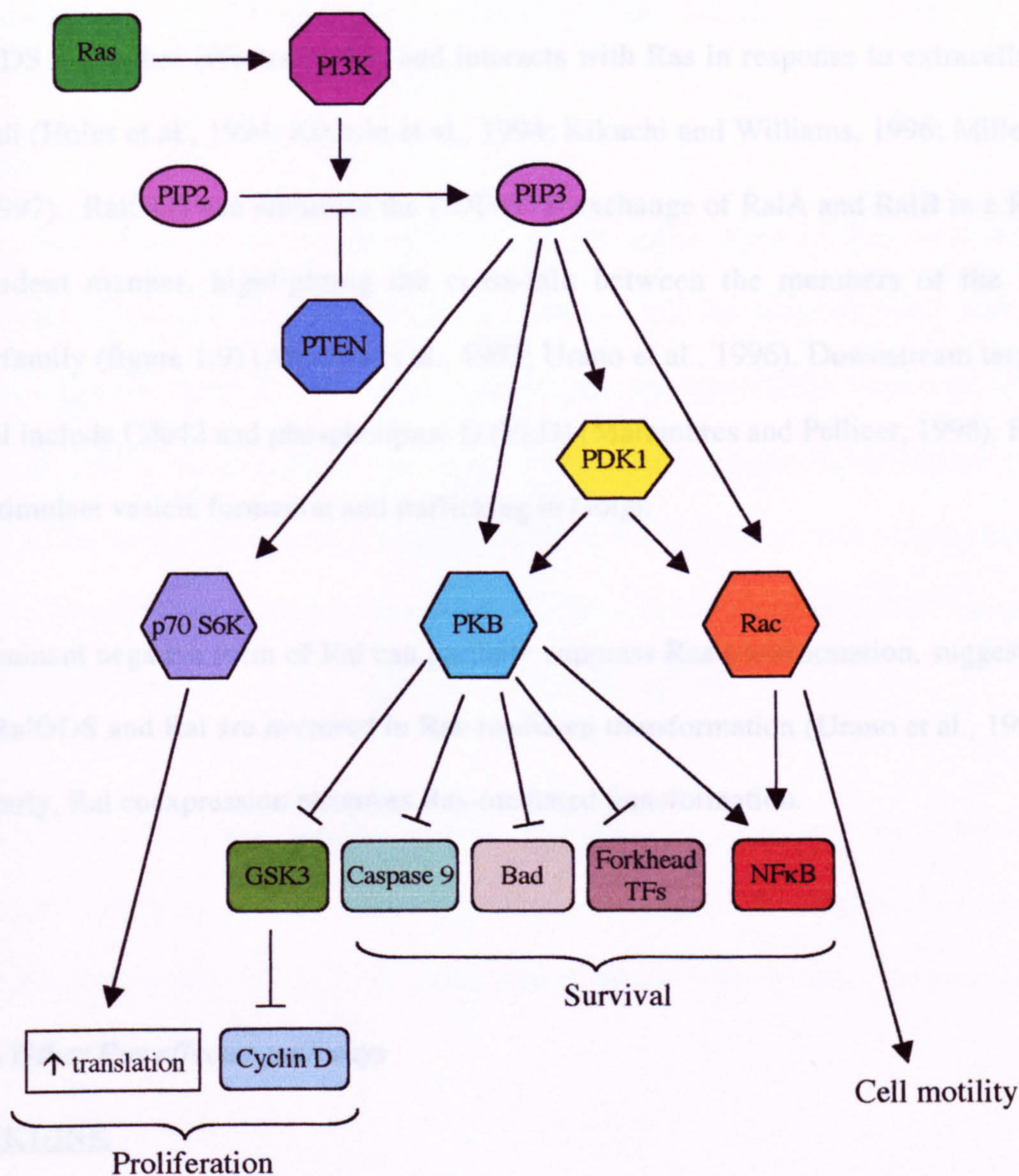


Figure 1.8. The Ras-PI3K signalling pathway. Ras stimulates PI3K which can then phosphorylate phosphatidylinositol (4,5) P_2 (PIP2) to phosphatidylinositol (3,4,5) P_3 (PIP3). PTEN is a phosphatase that reverses PI3K activity. PIP3 binds to and stimulates PKB, PDK1, Rac and p70 S6 kinase (p70 S6K). PDK1 further stimulates PKB and Rac. PKB inhibits Bad, caspase 9 and forkhead transcription factors (TFs), thereby preventing apoptosis. Both Rac and PKB can activate NFκB, further enhancing their survival signals. PKB phosphorylation of glycogen synthase kinase (GSK3) inhibits its activity, thereby allowing cyclin D accumulation and cell proliferation. p70 S6K activates the translation machinery, also resulting in increased proliferation. Rac is involved in cell motility and regulates actin filament formation.

1.7.5. Ral GDS pathway

RalGDS is another effector of Ras and interacts with Ras in response to extracellular stimuli (Hofer et al., 1994; Kikuchi et al., 1994; Kikuchi and Williams, 1996; Miller et al., 1997). RalGDS can stimulate the GDP/GTP exchange of RalA and RalB in a Ras-dependent manner, highlighting the cross-talk between the members of the Ras superfamily (figure 1.9) (Albright et al., 1993; Urano et al., 1996). Downstream targets of Ral include Cdc42 and phospholipase D (PLD) (Malumbres and Pellicer, 1998). PLD can stimulate vesicle formation and trafficking in Golgi.

A dominant negative form of Ral can partially suppress Ras transformation, suggesting that RalGDS and Ral are involved in Ras-mediated transformation (Urano et al., 1996). Similarly, Ral coexpression enhances Ras-mediated transformation.

1.7.6. Other Ras effector pathways

MEKK1/JNK

MEKK1 is a direct activator of the protein kinase JNKK (also known as SEK1/SAPKK1) which in turn activates the stress-activated protein kinase JNK/SAPK (Minden et al., 1994; Yan et al., 1994). JNK regulates c-Jun and ATF-2 activity by direct phosphorylation which results in increased transcription of target genes (Derijard et al., 1994; Gupta et al., 1995). MEKK1 stimulation of JNK and p38MAPK is associated with stress responses that result in apoptosis (figure 1.9) (Chen et al., 1996a; Verheij et al., 1996; Xia et al., 1995). Although *in vitro* data shows that MEKK1 binds Ras in a GTP-dependent manner and MEKK1 can be stimulated in a Ras-dependent

manner, it is not yet certain whether Ras activates MEKK1 directly *in vivo* (Lange-Carter and Johnson, 1994; Malumbres and Pellicer, 1998; Russell et al., 1995).

Nore1

Nore1 was identified as specifically binding the active form of Ras upon serum stimulation (Vavvas et al., 1998). Nore1 is related to, and can heterodimerize with, the tumour suppressor gene RASSF1, known to promote apoptosis (Ortiz-Vega et al., 2002; Vos et al., 2000). Nore1 has also been shown to promote apoptosis (Khokhlatchev et al., 2002) and is thought to do so in a manner similar to RASSF1 since both proteins bind to Mst1, a protein kinase involved in apoptosis (figure 1.9) (Graves et al., 1998; Lee et al., 1998). Mst1 activates caspase 3 upon FasL induced apoptosis.

Although the Ras-Nore1-Mst1 complex mediates apoptosis in cells exposed to tamoxifen, there is no change in Mst1 kinase activity following binding to Ras (Khokhlatchev et al., 2002). It is worth noting that this study was performed with overexpressed Ras.

AF-6

AF-6 and canoe, two structurally related proteins, bind to Ras in a GTP-dependent manner (Kuriyama et al., 1996). AF-6 function is unclear although it contains a motif shared among proteins that localize to specific cell-cell interaction sites. AF-6 is commonly found as a fusion protein to ALL-1 in a number of translocations present in acute lymphoblastic and myelocytic leukaemias (Prasad et al., 1993).

Unlike Raf, AF6 does not have a Ras binding domain (RBD) but instead has a Ras-association (RA) domain through which it interacts with Ras. The RA domain was proposed based on sequence and predicted structural similarities between RalGDS and AF6 (Ponting and Benjamin, 1996). Although the RBD of Raf and the RA domain of RalGDS have low levels of sequence homology, they have a similar tertiary fold (Geyer et al., 1997; Huang et al., 1997).

1.7.7. Cross-talk between Ras effectors

So far, Ras has been shown to activate discrete downstream pathways, such as the Raf-MAPK, the PI3K/PKB and the RalGDS/Ral pathways. In addition, however, these pathways both directly and indirectly cross-talk with each other. For example, as seen with Raf, the PI3K/PKB and the RalGDS/Ral pathways, in addition to their own signaling, further highlight the ability of the PI3K pathway to cross-talk with other pathways. Further highlighting the ability of the PI3K pathway to cross-talk with other pathways, other, was the observation that the PI3K pathway can cross-talk with the Raf-MAPK pathway through the stimulation of the Ets transcription factor, and the PI3K/PKB pathway through the inhibition of GSK-3 β , can synergize in inducing cyclin D1 expression and therefore proliferation. Thus it seems that the many downstream effector pathways of Ras have the potential to cross-talk with each other.

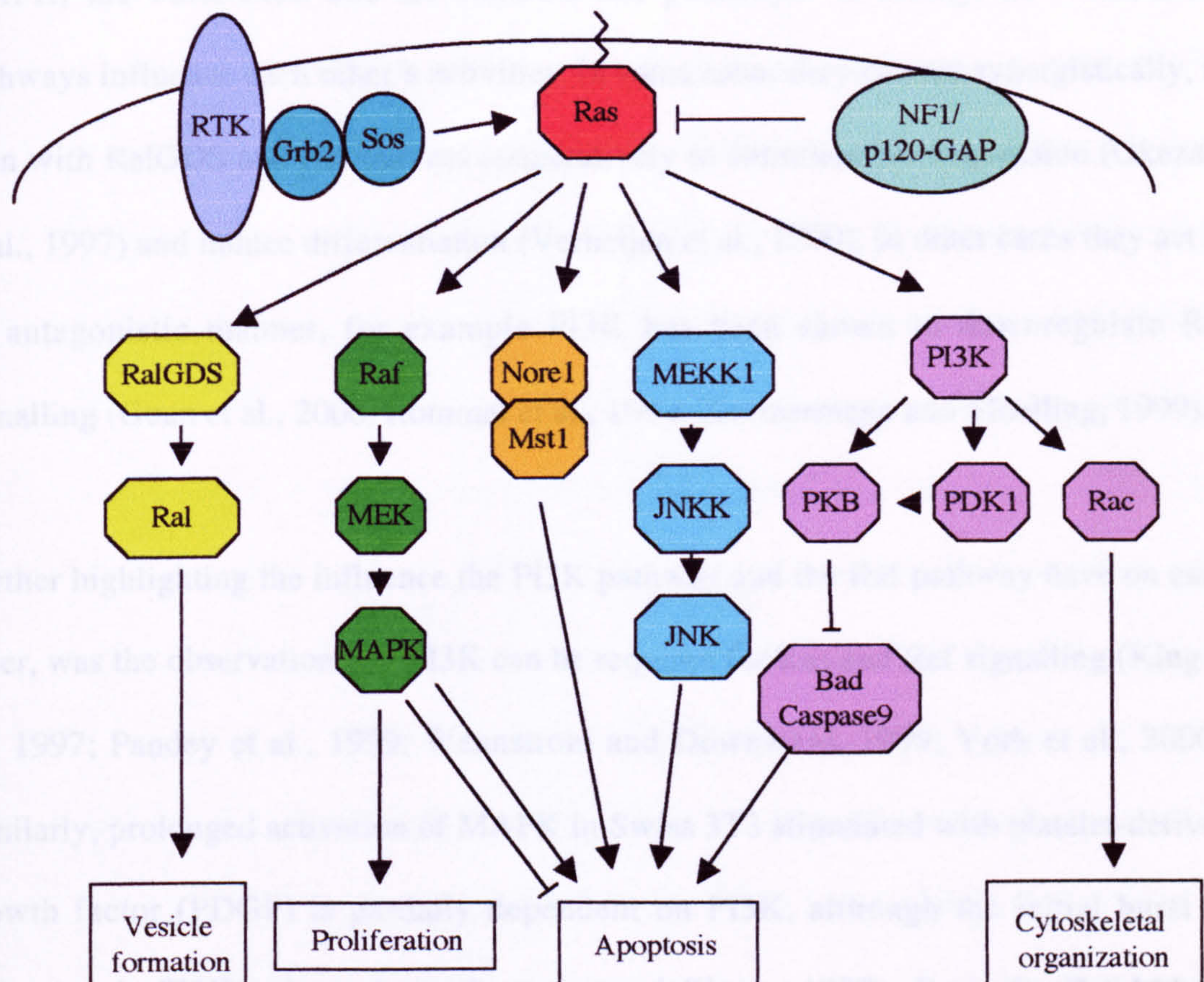


Figure 1.9. Summary of Ras downstream effector pathways. Ras is upstream of various effector pathways. Following ligand stimulation, the receptor tyrosine kinase (RTK) recruits Grb2 and Sos to the membrane. Sos is a GNEF that can activate Ras. NF1 and p120-GAP are GAPs that negatively regulate Ras by increasing its GTP hydrolysis rate. Once activated, Ras can stimulate a number of pathways: RalGDS activation results in Ral stimulation, leading to vesicle formation; Raf stimulates the MEK-MAPK pathway leading to proliferation, survival or apoptosis; Nore1 and the MEKK-JNK pathways stimulate apoptosis; and the PI3K stimulates cytoskeletal organization via Rac activation, and increased metabolism and survival via PKB activation.

1.7.7. Cross-talk between Ras effectors

So far, Ras has been shown to activate discrete downstream pathways, such as the Raf-MAPK, the PI3K-PKB and the RalGDS-Ral pathways. In reality, however, these pathways influence each other's activities. In some cases they can act synergistically, as seen with RalGDS and Raf that act cooperatively to stimulate Fos expression (Okazaki et al., 1997) and induce differentiation (Verheijen et al., 1999). In other cases they act in an antagonistic manner, for example PI3K has been shown to downregulate Raf signalling (Guan et al., 2000; Rommel et al., 1999; Zimmermann and Moelling, 1999).

Further highlighting the influence the PI3K pathway and the Raf pathway have on each other, was the observation that PI3K can be required for Ras and Raf signalling (King et al., 1997; Pandey et al., 1999; Wennstrom and Downward, 1999; York et al., 2000). Similarly, prolonged activation of MAPK in Swiss 3T3 stimulated with platelet-derived growth factor (PDGF) is partially dependent on PI3K, although the initial burst of activation is PI3K-independent (Grammer and Blenis, 1997). Both the Raf-MAPK pathway, through the stimulation of the Ets transcription factor, and the PI3K-PKB pathway through the inhibition of GSK, can synergize in inducing cyclin D1 expression and therefore proliferation. Thus it seems that the many downstream effector pathways of Ras interact with each other, adding further complexity to Ras signalling.

1.7.8. Ras and tumourigenesis

H-Ras and K-Ras genes were first discovered as viral oncogenes of the murine sarcoma viruses (MSV) Harvey-MSV and Kirsten-MSV (Ellis et al., 1981; Scolnick et al., 1975). The human homologs were later discovered as transforming genes from the T24 and EJ bladder carcinoma cell lines (Der et al., 1982; Goldfarb et al., 1982; Parada et al., 1982; Pulciani et al., 1982; Santos et al., 1982; Shih and Weinberg, 1982) and the third member of the family, N-Ras, was identified soon after (Hall et al., 1983; Shimizu et al., 1983). Further work showed that the functional difference between the normal Ras and its oncogenic allele is due to a single point mutation (Reddy et al., 1982; Tabin et al., 1982; Taparowsky et al., 1982). Ras was later found to be mutated in many human cancers (Bos, 1989), with a particularly high incidence in colon, lung and pancreatic carcinomas (Almoguera et al., 1988; Bos et al., 1987; Forrester et al., 1987; Rodenhuis et al., 1987). The mutated forms of Ras were shown to stimulate proliferation and transformation of cultured cells (Brown et al., 1984; Feramisco et al., 1984; Stacey and Kung, 1984).

Transforming Ras proteins are associated with alterations in their amino acids important for guanine nucleotide coordination. Mutant Ras proteins have abnormally high levels bound to GTP due to a decrease in their GTPase activity and/or an increase in nucleotide exchange rate, depending on the mutation. The most commonly mutated forms of Ras have single point mutations at residues 12, 13 or 61 (Bos, 1989; Taparowsky et al., 1983). Mutations in these residues reduce Ras GTPase activity and render the protein insensitive to GAPs with the net consequence that these Ras mutants are constitutively bound to GTP and therefore constitutively active (Adari et al., 1988; Martin et al., 1990; Trahey and McCormick, 1987).

Different Ras genes are preferentially mutated in different carcinomas. For example, K-Ras is commonly mutated in pancreatic cancers, colorectal malignancies and adenocarcinomas of the lung. H-Ras mutations are found in cutaneous squamous cell carcinomas and squamous head and neck tumours, whereas N-Ras are most frequently mutated in acute leukaemias and myelodysplastic syndromes (Rodenhuis, 1992). In accordance with the role of oncogenic K-Ras in human lung tumours, a transgenic mouse with an oncogenic form of *K-Ras* that can be spontaneously expressed developed lung tumours, thymic lymphomas and skin papillomas (Johnson et al., 2001).

Ras involvement in tumorigenesis does not only occur through point mutations, it can also arise from overexpression, as seen in tumour formation following N-Ras overexpression in transgenic mice (Mangues et al., 1992). Whilst overexpression of GAPs can prevent transformation by Ras overexpression, it has no effect on the transformation potential of Ras mutants with mutations in their GTPase region.

Proteins involved in Ras regulation or its downstream pathways have also been found to be mutated in cancers. Overexpression of ErbB2 or EGFR is common in breast, lung, pancreas and colon cancers (Yarden and Sliwkowski, 2001) and it has recently been shown that EGFR is mutated in non-small cell lung cancer (Lynch et al., 2004; Paez et al., 2004). Loss of negative regulators of Ras, such as NF1, can cause tumours with increased Ras activity (Basu et al., 1992). The downstream effector of Ras, B-Raf, is also mutated in most human melanomas as well as in many other tumours (Davies et al., 2002) and another Ras effector, PI3K, is amplified in ovarian tumours and has recently been found to have activating mutations in various tumours (Samuels et al., 2004).

Activated Ras can also be detected in premalignant lesions, suggesting a role in tumour initiation. It is possible that Ras mutations arise in healthy individuals and remain silent until other genetic alterations occur, as seen in colon carcinogenesis (Kinzler and Vogelstein, 1996).

Oncogenic Ras is not only more active than its normal counterpart, it can signal differently as well. Ras signalling in tumours is persistent compared to the temporally fluctuating signal found in normal cells and as a consequence oncogenic Ras can activate some effectors effectively, such as PI3K, whilst normal Ras can only weakly activate PI3K (McCormick, 1999). Thus, normal endogenous Ras can stimulate PI3K to activate PKB but has no effect on lamellipodium formation, whereas ectopically expressed activated Ras can induce both PKB activation and lamellipodium formation (van Weering et al., 1998).

Unlike its viral counterpart, the human Ras oncogene is not sufficient to transform cells in culture (Land et al., 1983; Newbold and Overell, 1983; Ruley, 1983). Transformation of rodent primary cells requires cooperation of a second oncogene, such as the adenoviral E1A protein or Myc (Land et al., 1983; Ruley, 1983). The tumour promoting function of an oncogene is frequently balanced by an inhibitory effect which must be overcome by additional mutations to allow tumourigenesis (Evan and Vousden, 2001). For example, in REFs, high Ras activity without a cooperating oncogene results in arrest at the G2 phase of the cell cycle, and Myc deregulation results in apoptosis (Evan et al., 1992). Human and rodent cells differ in their susceptibility to transformation: human cells are not transformed by two cooperating oncogenes, they require a multistep

process for the development of human tumours, as seen with colorectal cancers (Hahn and Weinberg, 2002; Kinzler and Vogelstein, 1996).

1.7.9. Ras regulation of cell cycle

Proliferation

The first indication that Ras was involved in mitogenesis following serum- or growth factor-stimulation came with studies in which Ras activity was inhibited with a neutralizing antibody, dominant negative form of Ras, p120-GAP or anti-sense oligonucleotides (Chang et al., 1991; Feig and Cooper, 1988; Kung et al., 1986; Monia et al., 1992). Ras can also induce DNA synthesis in quiescent cells; Ras signalling is required at several points during G1 for the stimulation of quiescent cells into S phase (Mulcahy et al., 1985; Stacey and Kazlauskas, 2002).

One important function of activated Ras is to overcome the effects of the tumour suppressor Rb that prevents the cell entry into S phase (Lee et al., 1999b; Mittnacht et al., 1997; Peeper et al., 1997). Ras can do so by stimulating cyclin D1 expression. Cyclin D1 is a major player in cell cycle progression in G1 as described in section 1.2. Briefly, cyclin D activates Cdk4/6 which phosphorylate Rb, rendering it unable to inhibit E2F1, ultimately leading to the progression of the cell cycle from G1 to S phase. Although cyclin D1 is necessary for Ras stimulation of cell cycle progression, it is not sufficient for its transforming activity (Aktas et al., 1997; Liu et al., 1995).

Cyclin D1 activation by Ras is dependent on the Raf-MAPK pathway (Lavoie et al., 1996). A dominant negative form of Ras leads to reduced levels of cyclin D1, accumulated hypophosphorylated Rb and G1 arrest (Leone et al., 1997; Peeper et al., 1997). Cyclin D1 is also regulated by the PI3K pathway during the later stages of G1 (Gille and Downward, 1999; Jones and Kazlauskas, 2001): the PI3K-PKB pathway stabilizes cyclin D1 by inhibiting its GSK-mediated phosphorylation (figure 1.8) (Diehl

et al., 1998). Additionally, PI3K mediates p27 downregulation that occurs in late G1, allowing the cell to proceed into S phase (Takuwa and Takuwa, 1997). The MAPK pathway also seems to be involved in p27 degradation (Kawada et al., 1997). MAPK-dependent degradation of the inhibitor p27 results in activated cyclin E, leading to increased proliferation (see section 1.2) (Aktas et al., 1997; Kawada et al., 1997; Leone et al., 1997; Takuwa and Takuwa, 1997). Therefore, in order to progress through the cell cycle, Ras needs more than one effector pathway (Marshall, 1999).

Cell cycle arrest

As mentioned above in section 1.7.8, activated Ras leads to cell cycle arrest in rodent primary cells unless it is coexpressed with a cooperative oncogene (Franza et al., 1986; Hicks et al., 1991). The Ras-Raf pathway can also induce cell cycle arrest in some human cancer cell lines (Ravi et al., 1999; Ravi et al., 1998b). It has been proposed that Ras cooperation with Myc, E1A, dominant negative p53 or the SV40 T antigen is due to the fact that these oncogenes can prevent Ras induction of cell cycle arrest, allowing Ras to induce proliferation (Hicks et al., 1991; Lloyd et al., 1997).

The main manner in which Ras can induce cell cycle arrest is by inducing expression of p21 and the p16 member of the INK family (see sections 1.2 and 1.4) (Lin et al., 1998; Lloyd et al., 1997; Malumbres et al., 2000; Pumiglia and Decker, 1997; Serrano et al., 1997). In primary cells, Ras activation of p53 induces cell cycle arrest that is indistinguishable from senescence, as seen by flat morphology and other senescence markers (Serrano et al., 1997).

Thus Ras can induce a number of opposing effects on the cell cycle: from proliferation to arrest, and even in some cases, differentiation (Noda et al., 1985). How Ras decides which pathway to induce is still unclear but is likely to be dependent on the presence of cooperating oncogenes. It has also been suggested that high levels of activated Ras can induce arrest, whereas lower levels of Ras induces proliferation (Sewing et al., 1997).

1.7.10. Choice of response: to die or not to die?

There have been many reports that Ras can inhibit apoptosis and promote survival. However, paradoxically, there have also been reports that Ras can induce apoptosis. The effect of Ras on survival versus apoptosis depends largely on cell types and other signalling pathways occurring at the time. In normal cells, high levels of activated Ras are more likely to induce apoptosis whereas activated Ras in transformed cells is more likely to mediate survival.

Induction of apoptosis

Ras can induce apoptosis in a number of different cell lines, such as lymphocytes and fibroblasts. In fibroblasts, Ras has been involved in the apoptotic response following various types of stress, such as tamoxifen treatment, tumour necrosis factor (TNF) treatment and forced Myc expression during serum starvation (Kauffmann-Zeh et al., 1997; Trent et al., 1996; Vater et al., 1996). In lymphocytes, Ras can be activated by the IL-2 receptor or by an antigen receptor; following stimulation, Ras has been shown to induce either proliferation or apoptosis. The choice of response was largely dependent on the other stimuli acting in the cells simultaneously (Gomez et al., 1996; Gomez et al., 1997; Latinis et al., 1997).

Ras-induced apoptosis has been shown to be mediated by the Raf-MAPK pathway (figure 1.9) and, in accordance with this, a dominant negative form of Raf can block the ability of Ras to induce apoptosis (Fukasawa and Vande Woude, 1997; Kauffmann-Zeh et al., 1997; Navarro et al., 1999). p53 is also thought to be involved in apoptosis following Ras signalling as Ras is no longer able to induce apoptosis following the loss of p53 (Fukasawa and Vande Woude, 1997; Nikiforov et al., 1996; Vater et al., 1996).

Interestingly, the phase of the cell cycle is thought to influence the Ras decision to mediate apoptosis: MAPK activation in Swiss 3T3 fibroblasts results in apoptosis only in cells found in S phase at the time of the signal, if cells are in other parts of the cell cycle, MAPK induces arrest (Fukasawa and Vande Woude, 1997).

Ras can stimulate apoptosis in a Raf-independent manner as well: constitutively activated Ras can stimulate the MEKK-JNK pathway (see section 1.7.5), thereby stimulating apoptosis (figure 1.9) (Xia et al., 1995). However, Ras alone is not sufficient to fully activate JNK (Rausch and Marshall, 1997).

As mentioned above, the Ras effector protein Nore1 is implicated in apoptosis (figure 1.9) (Khokhlatchev et al., 2002). Induction of Nore1 by Ras stimulates apoptosis in an Mst1 dependent manner (Graves et al., 1998; Khokhlatchev et al., 2002; Lee et al., 1998). K-Ras is more effective than H-Ras at promoting apoptosis (Khokhlatchev et al., 2002). Since K-Ras can preferentially activate the Raf-MAPK pathway rather than the PI3K pathway (Yan et al., 1998), it is possible that K-Ras signalling to Nore1 might not be significantly counterbalanced by the PI3K-mediated survival signals, and therefore K-Ras has a greater propensity to induce apoptosis.

Signalling survival

In epithelial cells and myeloid cells, Ras stimulation mostly results in survival signals (Downward, 1998b). Ras has been shown to prevent apoptosis in epithelial cells following E1A overexpression and detachment from the extracellular matrix (Frisch and Francis, 1994; Lin et al., 1995; Rak et al., 1995).

The main Ras effector pathway mediating the survival signals is the PI3K pathway: activated PI3K or Akt can abrogate apoptosis whilst inhibition of the PI3K pathway can enhance apoptosis (figure 1.9) (Cox and Der, 2003; Datta et al., 1999; Downward, 1998b; Dudek et al., 1997; Gire et al., 2000; Kauffmann-Zeh et al., 1997; Yao and Cooper, 1995). PI3K can prevent apoptosis in various ways (figure 1.8). Firstly it stimulates PKB phosphorylation of the pro-apoptotic Bcl-2 family member Bad at serine 136. Once phosphorylated, Bad binds 14-3-3, forming an inactive complex instead of sequestering the anti-apoptotic proteins Bcl-X_L and Bcl-2 (Datta et al., 1997; del Peso et al., 1997; Zha et al., 1996). Bcl-X_L and Bcl-2 are therefore free to inhibit apoptosis (see section 1.3). Secondly, PI3K can phosphorylate caspase 9, preventing its activation by cytochrome C. This suggests that PI3K can block apoptosis downstream of cytochrome C release (Cardone et al., 1998). Thirdly, PKB influences survival by phosphorylating the Forkhead transcription factors. The Forkhead transcription factors phosphorylation leads to their sequestration in the cytoplasm where they are inactive (Biggs et al., 1999; Brunet et al., 1999). Forkhead transcription factors can transcribe many genes involved in apoptosis, such as *Bax* and *FasL* (Brunet et al., 1999; Miyashita et al., 1994b; Miyashita and Reed, 1995; Zhan et al., 1994). Lastly, PI3K has been shown to suppress apoptosis through activation of NF- κ B. PI3K-mediated activation of Rac can stimulate NF- κ B to protect cells from death (figure 1.8) (Joneson and Bar-Sagi, 1999; Sulciner et al., 1996). In fibroblast cells, inhibition of NF- κ B has lead to Ras induction of apoptosis in a p53-independent manner, suggesting that NF- κ B is necessary for Ras-mediated survival (Mayo et al., 1997b). PI3K can also stimulate NF- κ B activity by the PKB pathway: PKB has been shown to phosphorylate and inactivate the NF- κ B inhibitor, I κ B (figure 1.8) (Ozes et al., 1999). NF- κ B protects cells from apoptosis by promoting the transcription of anti-apoptotic genes such as the inhibitors

of apoptosis proteins (IAP) and Bcl2-family members, described in section 1.3.4 (Chu et al., 1997).

Although PI3K is the main Ras-induced pathway involved in survival, the Raf-MAPK has also been shown to be involved in survival signalling in some circumstances (figure 1.9). For example, MAPK protects neuronal cells from death following neurotrophic factor withdrawal (Xia et al., 1995) and protects fibroblasts from apoptosis following loss of attachment (Le Gall et al., 2000). As with PI3K, mitochondrial Raf and MEK signalling have also been involved in Bad phosphorylation, disrupting Bad-Bcl2 interactions and thereby allowing Bcl-2 to inhibit apoptosis (Fang et al., 1999b; Scheid et al., 1999; Wang et al., 1996). As mentioned above, the downstream substrate of the Raf-MAPK pathway, RSK, can phosphorylate CREB, thereby activating it. The transcription factor CREB is involved in survival pathways, showing another pathway by which Ras-Raf-MAPK can mediate survival (Bonni et al., 1999; Finkbeiner, 2000).

The Raf-MAPK pathway has also been implicated in promoting survival in IL-3 dependent hematopoietic cells (Kinoshita et al., 1997; Terada et al., 2000), although PKB is still required for full survival signals in these cells: dominant negative forms of PKB and the PI3K-inhibitor wortmannin suppress Raf-mediated survival (von Gise et al., 2001).

Thus Ras can induce both survival and apoptosis, depending on the cell type and cell context. Whereas the PI3K pathway is mainly involved in survival signalling and the Nore1 pathway is mainly involved in apoptotic signalling, the Raf-MAPK pathway is less straightforward and can be involved in the induction of either response (figure 1.9).

1.8. Ras & p53

Ras induces cell cycle arrest in a p53-dependent manner

As mentioned in section 1.7.9, in the absence of cooperating oncogenes, Ras can induce cell cycle arrest and this arrest is mediated by the Cdk inhibitors p21 and p16. Interestingly, p21 induction by Ras is mediated by p53: activated Ras in primary mouse or human fibroblasts leads to increased levels of p53, which then stimulates its downstream target p21 to induce cell cycle arrest. Data confirming the role of p53 in mediating Ras-dependent cell cycle arrest comes from the observations that *p53* knockout MEFs are highly susceptible to Ras transformation and that Ras can induce transformation in cells harbouring p53 dominant negative mutants (Hicks et al., 1991; McMahon and Woods, 2001). Thus, p53 plays a major role in preventing Ras-mediated transformation by inducing cell cycle arrest in response to activated Ras. In some cases, loss of p21 can also predispose Ras to transformation, suggesting that p21 plays an important role in inhibiting Ras-induced transformation (Missero et al., 1996; Topley et al., 1999).

Arf

Ras stimulation of p53 occurs in an Arf-dependent manner since Ras transformation activity is inhibited by Arf overexpression in *p53* wild-type but not in *p53*-null cells (figure 1.10) (Kamijo et al., 1997; Pomerantz et al., 1998). In accordance with a role of Arf in mediating Ras transformation, *Arf* knockout MEFs are highly susceptible to Ras transformation (Kamijo et al., 1997). As mentioned in section 1.4.5, Arf can stabilize p53 levels by inhibiting the p53-negative regulator Mdm2, thereby increasing p53 activity. It is still unclear how Ras induces Arf expression, but it is thought to involve

the transcription factors Myc, E2F1 or DMP1 (Bates et al., 1998; Dimri et al., 2000; Inoue et al., 1999). DMP1 can bind the Arf promoter *in vitro* and induces Arf-dependent arrest mediated by p53 (Inoue et al., 1999). DMP1 is required for full Arf and p53 stimulation in response to activated Ras and *DMP1*-null fibroblasts are susceptible to Ras-mediated transformation (Inoue et al., 2000).

PML

Following Ras-induced cell cycle arrest, PML is upregulated. Forced expression of PML is sufficient to induce upregulation of p53, p16 and premature arrest, all similar phenotypes to those seen following Ras activation (Ferbeyre et al., 2000; Pearson et al., 2000). As mentioned in section 1.4.5, upon Ras stimulation, p53 associates with PML in the nuclear bodies (NBs) and forms a trinary complex with CBP. p53 gets acetylated by CBP, following which its transactivation activity is stimulated (figure 1.10) (Fogal et al., 2000). PML is necessary for Ras-induced senescence as *PML*-null fibroblasts no longer undergo senescence in the presence of activated Ras (Pearson et al., 2000; Ries et al., 2000). Although PML is not necessary for the p53 protein level increase observed after Ras activation, it is required for p53-dependent transactivation of *p21*, and therefore in Ras-mediated in cell cycle arrest.

Mdm2

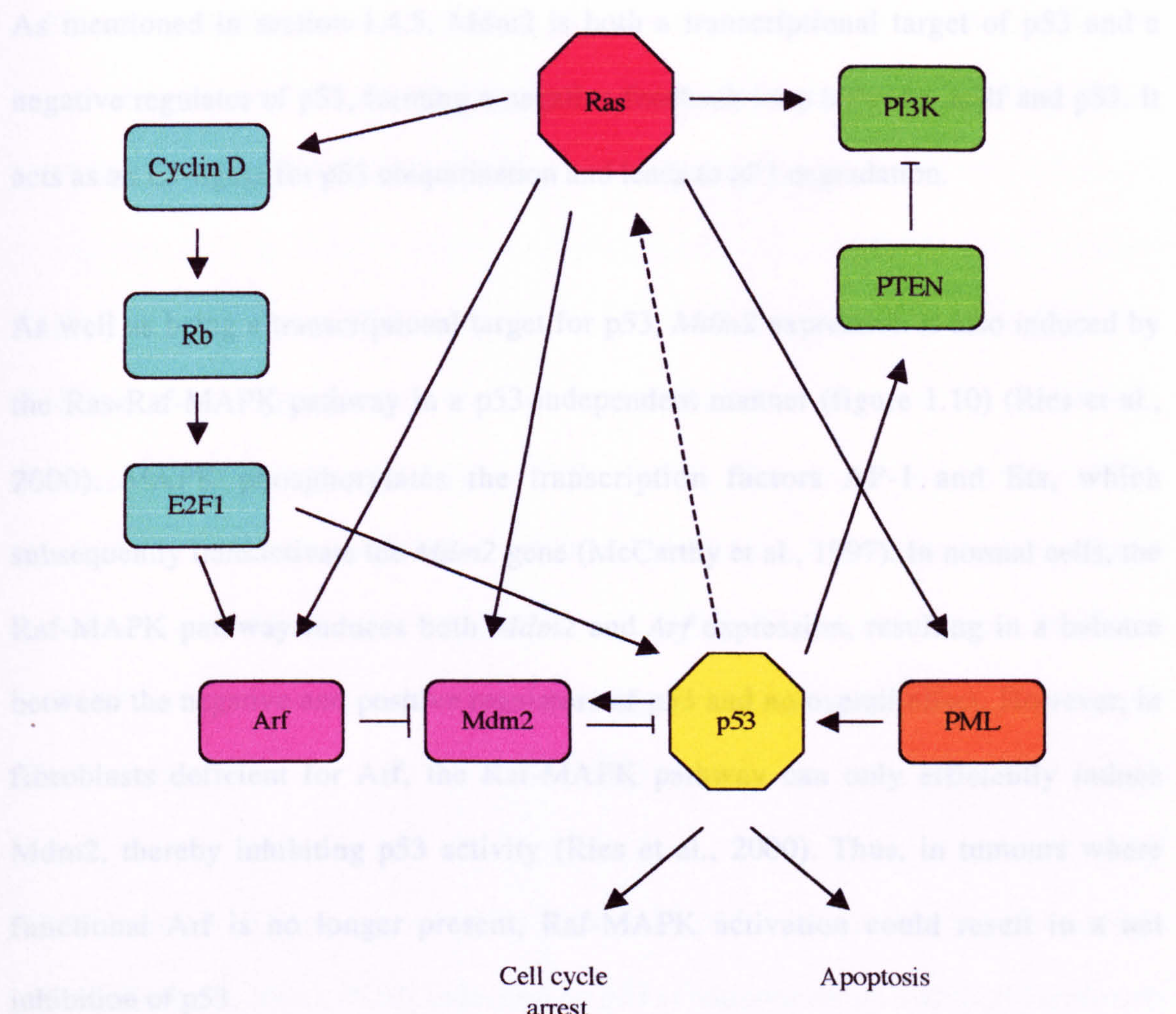


Figure 1.10. Ras and p53 crosstalk. Ras can stimulate p53 activity via various pathways. It can induce cyclin D1 expression, resulting in upregulation of E2F1 activity. E2F1 can then transactivate the *Arf* gene, thereby stabilizing p53. E2F1 can also synergize with p53 to induce apoptosis. Ras can also stimulate Arf expression in an E2F1-independent manner, possibly through the DMP1 transcription factor. Ras can negatively regulate p53 by inducing *Mdm2* expression. By stimulating PML, Ras can increase p53-mediated transactivation and induce cell cycle arrest. *PTEN* phosphatase is a p53 target gene and can reverse Ras-mediated PI3K activity. p53 has been shown to increase Ras activity, either by directly transactivating the *H-ras* gene or by inducing the expression of growth factors upstream of Ras (see text for details).

Mdm2

As mentioned in section 1.4.5, Mdm2 is both a transcriptional target of p53 and a negative regulator of p53, forming a negative feedback loop between itself and p53. It acts as an E3-ligase for p53 ubiquitination and leads to p53 degradation.

As well as being a transcriptional target for p53, *Mdm2* expression is also induced by the Ras-Raf-MAPK pathway in a p53-independent manner (figure 1.10) (Ries et al., 2000). MAPK phosphorylates the transcription factors AP-1 and Ets, which subsequently transactivate the *Mdm2* gene (McCarthy et al., 1997). In normal cells, the Raf-MAPK pathway induces both *Mdm2* and *Arf* expression, resulting in a balance between the negative and positive regulators of p53 and no overall effect. However, in fibroblasts deficient for Arf, the Raf-MAPK pathway can only efficiently induce Mdm2, thereby inhibiting p53 activity (Ries et al., 2000). Thus, in tumours where functional Arf is no longer present, Raf-MAPK activation could result in a net inhibition of p53.

E2F1

Ras can increase cyclin D1 activity in two manners: the Ras-Raf-MAPK pathway can induce cyclin D1 expression and the Ras-PI3K-PKB pathway can stabilize cyclin D1 expression (see section 1.7.9) (Cheng et al., 1998; Diehl et al., 1998; Lavoie et al., 1996). High levels of cyclin D1 can stimulate Cdk4 and Cdk6 to phosphorylate Rb (see section 1.2.1). Phosphorylated Rb is no longer able to bind to the E2F1 transcription factor, and dissociated E2F1 is subsequently free to transactivate its target genes. One of the E2F1 target genes is Arf (Dimri et al., 2000; Zhu et al., 1999). Thus, E2F1 can stimulate p53 activity by directly inducing Arf expression (figure 1.10). It has been

shown that E2F and Ras can synergize in their induction of Arf (Berkovich et al., 2003). Additionally, E2F1 can also stimulate p53 apoptotic activity in a transcriptionally-independent manner; this stimulation requires the cyclin A binding site of E2F1 (figure 1.10) (Hsieh et al., 2002).

PTEN

PTEN is a lipid phosphatase that can antagonize PI3K function by specifically dephosphorylating PtdIns(3,4,5)P₃ to PtdIns(4,5)P₂ (Maehama and Dixon, 1998): tumour cell lines lacking PTEN and cell lines derived from *PTEN*-knockout mice have increased levels of PtdIns(3,4,5)P₃ (Katso et al., 2001). The loss of function mutations of *PTEN* and somatic deletions of the gene in tumours strongly support its role as a tumour suppressor.

PTEN is also a p53 target gene and is induced by p53 wild-type but not p53 mutants (Stambolic et al., 2001). PTEN induction by p53 is required for p53-mediated apoptosis in immortalized mouse embryo fibroblasts. Thus p53 transactivation of PTEN can inhibit Ras activation of the PI3K pathway.

p53 can activate Ras

It has been reported that p53 can stimulate Ras expression. A conserved p53 response element has been found in the first intron of H-Ras gene and this response element can confer the ability of H-Ras to be transactivated in a p53-dependent manner (figure 1.10) (Deguin-Chambon et al., 2000). Further studies have showed that p53 can induce sustained MAPK activation, but in this case the p53 target responsible for this activation

lies upstream of Ras, possibly at the level of heparin-binding EGF-like growth factor (HB-EGF) (Lee et al., 2000).

In all the cases described in this section, Ras can activate p53 to specifically induce cell cycle arrest. It is not uncommon for the transforming activity of an oncoprotein to be counterbalanced by an inhibitory effect; it acts as a fail-proof system for cells to prevent the formation of tumours.

1.9 Aim of study

The ASPP1 and ASPP2 proteins have recently been shown to be major players in p53 regulation. Unlike other p53 regulators, they can specifically enhance p53 pro-apoptotic activity. The activity of the ASPP proteins clearly has to be regulated in a stringent manner to prevent apoptosis occurring in an uncontrollable manner.

My objectives were to increase our understanding of how ASPP1 and ASPP2 are regulated, and therefore how p53 apoptotic activity is regulated. I have addressed this issue by investigating an ASPP2 mutant that is partially defective in inducing p53 pro-apoptotic activity. This mutant has its amino-terminal region deleted and, since this region does not contain the p53-interaction site, I consequently hypothesised that this amino-terminal region of ASPP2 may contain a regulatory domain necessary for its full activity.

The first section of my thesis analyzes this amino-terminus region and the Ras-association domain it contains. I investigate whether Ras binds ASPP1 and ASPP2 and whether this has any effect on ASPP activity. Ras is upstream of various effector pathways, many of which are activated by phosphorylation cascades. In the second part of my thesis, I therefore examine the possibility that ASPP activity is influenced by one of the downstream effector pathways of Ras. Finally, I briefly probe into other ways in which ASPP1 and ASPP2 activity could be regulated.

Chapter 2

Materials & Methods

2.1 Materials

2.1.1. Reagents

All chemicals, unless otherwise stated, were obtained from BDH Chemicals, UK. All radio-isotopes, autoradiography films (Hyperfilm), ECL (Enhanced Chemi-Luminescence) reagents were purchased from Amersham Pharmacia Biotech (UK). All restriction enzymes, their buffers, were purchased from New England Biolabs (UK). All tissue culture dishes and flasks were from Greiner (UK).

The Luciferase Assay System Kit and the TNT® T7 Quick Coupled Transcription/Translation System Kit was purchased from Promega (WI, USA). The Tet inducible cell system was purchased from CLONTECH (CA, USA) and the QIAGEN Plasmid Mega Kit was purchased from Qiagen (UK). The In Situ Cell Death Detection Kit, Fluorescein was purchased from Boehringer Mannheim (UK).

5x Agarose Sample Buffer

50% (w/v)	Sucrose
100 mM	EDTA, pH 8.0
0.1% (w/v)	Bromophenol blue
0.1% (w/v)	Xylene cyanol FF

Agarose gel for DNA electrophoresis

Agarose powder (GibcoBRL, UK) was weighed and dissolved in 1X TAE buffer at an appropriate concentration. The mixture was heated in a microwave oven to dissolve the agarose and the solution allowed to cool to 40°C. Ethidium bromide was added to a final concentration of 20µg/ml. The agarose solution was poured into a casting tray with the required comb and was left to solidify at room temperature.

Ammonium Persulphate (APS)

10% (w/v) stock solution was prepared in water, and stored at -20°C in single-use aliquots.

Ampicilin Stock

5 g of the antibiotic was dissolved in 25 ml sterile distilled water and 25 ml of ethanol making a 100 mg/ml stock solution in 50% ethanol. This was stored at -20°C as aliquots.

Blocking Solution

10% (w/v) fat-free milk (Marvel, UK) was prepared in phosphate buffered saline (1X TBS-T).

Calcium chloride

A 2.5 M solution was made up by dissolving 36.75 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in distilled water, filter-sterilised and stored at room temperature. This was used solely for calcium phosphate transfection.

Cisplatin

The stock concentration was made to 1mg/ml with distilled water and cisplatin was used at a final concentration of $3\text{ }\mu\text{g/ml}$. The cisplatin is provided by Pharmacia via St Mary's Hospital pharmacy.

COMpleteTM protease inhibitor cocktail

1 x COMpleteTM protease inhibitor (Boehringer Mannheim, UK) tablet was dissolved in 2.0 ml of sterilised distilled water as a 20x stock solution that is stable at -20°C for 12 weeks.

Coomassie Staining Solution

82% (v/v) Ethanol

18% (v/v) Acetic acid

1/10000 (w/v) Brilliant blue R (Sigma)

Alternatively Gelcode was used (Pearce) according to the manufacturer's directions.

Cyclohexamide

A stock solution of 50mg/ml was prepared by dissolving the powder in ethanol. It was stored at -20°C . The working solution was used at a concentration of 50 $\mu\text{g/ml}$.

DAPI staining solution

A stock solution of 10 mg/ml was made by dissolving 4'-6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Sigma, MO, USA) in water and then adding 10 x PBS to make a 1 $\mu\text{g/ml}$ working solution in 1 x PBS. For visualisation of mycoplasma, 0.1 $\mu\text{g/ml}$ DAPI solution was used as described (Russell et al., 1975).

Daunorubicin

Daunorubicin was purchased from Sigma (UK) and was used at a final concentration of 300ng/ml.

Destain buffer

50%	water
40%	methanol
10%	acetate

Dialysis buffer

50mM	Tris-Hcl, pH 7.5
100mM	NaCl
5mM	MgCl ₂
Immediately before use add	
1mM	DTT

Doxorubicin

The solution was purchased from Pharmacia (UK) via the St Mary's Hospital pharmacy at a concentration of 0.5mg/ml. It was used at a final concentration of 1 μ M.

EDTA Solution

A 0.5M $C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$ (EDTA) stock solution was made up by dissolving 18.6 g of EDTA in 70 ml distilled water. The pH was adjusted to pH 8.0 with NaOH and the volume made up to 100 ml with distilled water.

Ethidium Bromide

A 10 mg/ml stock solution was prepared by dissolving 0.2 g ethidium bromide in 20 ml of distilled water the stored at 4 °C in the dark.

Fixer Solution

50% (v/v)	Ethanol
10% (v/v)	Acetic acid

G-418 (Geneticin® or Neomycin)

A stock solution was prepared under sterile conditions by dissolving 5 mg of the powered G-418 Geneticin® (G-418 Sulphate; Life Technologies Ltd, UK) in 50 ml of DMEM to make a 100 mg/ml stock which was stored at 4 °C.

Giemsa staining solution

Giemsa staining solution (Pierce, UK) was diluted 1:20 in distilled water as recommended.

GST lysis buffer

50mM	Tris-HCl, pH 7.5
50mM	NaCl
5mM	MgCl ₂
Immediately before use add	
1mM	DTT
1mM	PMSF

2X HBS (HEPES buffered saline)

39 mM	50 ml of 390mM stock (9.25 g in 100 ml) HEPES (<i>made fresh</i>)
10 mM	50 ml of 100mM stock (0.744 g in 100 ml) KCl
280mM	50 ml of 2.8 M stock (16 g in 100 ml) NaCl
1.5 mM	50 ml of 15mM stock (0.213 g in 100 ml) Na ₂ HPO ₄
12mM	50 ml of 120mM stock (2.16g/100ml) Sucrose

The solution was adjusted to pH 6.90-7.3 with NaOH and filter sterilised for transfections. These were stored in 50 ml aliquots at -20 °C for long term storage and 4°C for short term storage.

5x *In vitro* kinase buffer

250 mM	Tris-Hcl pH 7.5
1.35 M	sucrose
0.5 mM	EGTA
0.5% v/v	mercaptoethanol

Loading buffer

50mM	Tris-HCl pH7.6
50mM	NaCl
5mM	MgCl ₂
10mM	EDTA

On the day add

5mM	DTT
2μCi	[³ H] GDP or [³ H] GTP (10 ci/mmol, 1μCi/ml), Amersham Pharmacia

Luciferase Assay System

This assay system was purchased from Promega (WI, USA). The solutions were made up according to the manufacturer's directions and stored at -20 °C and allowed to equilibrate to room temperature before use.

LY294004

The powder was ordered from Calbiochem and a 50 mM stock concentration was made by dissolving the powder in DMSO. The working solution was 10 μM.

Membrane buffer

50mM	Tris pH 7.9
0.5%	NP40 (non-ionic detergent)
0.1%	Na Deoxycholate
0.05%	SDS
20mM	O-octyl glucopyranoside (non-ionic detergent)
0.5mM	EDTA
0.5mM	EGTA
5mM	Na pyrophosphate
25mM	Na B-glycerophosphate
10%	glycerol
0.1%	B-meraptoethanol
On the day add	
1mM	Na ₃ VO ₄
protease inhibitors	

Methanol/Acetone

50% (v/v)	Methanol
50% (v/v)	Acetone

Mowiol

6ml of glycerol, 6 ml of distilled water and 2.4g of Mowiol 4-88 (Calbiochem) were mixed in a 50 ml falcon tube. The mixture was vortexed and then shook for 2 hours. 12 ml of 200 mM Tris-HCl, pH 8.5 was added and the solution was incubated at 50°C with occasional mixing until the Mowiol dissolves (approximately 3 hours). The solution was filtered through 0.45 µm syringes and stored in aliquots at 4°C. Before each aliquot's use, 2.5% w/v 1,4-diazabicyclo-[2.2.2]octane (DABCOA, Sigma, UK) was added and the solution was vortexed for 30 minutes to dissolve and left overnight at 4°C for the bubbles to disappear.

NET Buffer

150 mM	NaCl
50 mM	Tris-HCl, pH 7.5
5 mM	EDTA, pH 8.

Nonidet P40 (NP40) Lysis Buffer

100 mM	NaCl
50 mM	Tris-HCl, pH 8.0
1 mM	EDTA, pH 8.0
1% (v/v)	NP40
1 mM	PMSF (Sigma)
supplemented with phosphatase /protease inhibitors	

Para-formaldehyde Fixing Solution

A 4 % (w/v) of Para-formaldehyde was first dissolved in water in the fume-hood and then 10 X PBS added till the final concentration of PBS was 1 x. The pH was adjusted to 7.0 with 1 M HCl and the solution was stored at room temperature.

PD98059

A 50 mM stock concentration was prepared by dissolving PD98059 (Calbiochem) into DMSO. The final concentration was used at 100 μ M.

Phosphatase inhibitor cocktails I and II

The solutions were purchased from Sigma. They were stored at 4°C and were used 1:100 as directed by the manufacturer's directions.

Phosphate Buffered Saline (PBS)

12.5 mM	NaCl
1 mM	Sodium dihydrogen phosphate, NaH_2PO_4
1.6 mM	Disodium dihydrogen phosphate, Na_2HPO_4

The pH was adjusted to 7.0 and autoclaved. The solution was prepared by the Ludwig washroom team.

PMSF (phenylmethanesulphonylfluoride)

A 20 mg/ml (100x) stock solution of PMSF $C_7H_7FO_2S$ (Sigma, UK) was made up in iso-propanol and stored in 1 ml aliquots at $-20\text{ }^{\circ}\text{C}$.

Ponceau S Staining Solution (10 X)

- 5% (v/v) Acetic acid
- 2% (v/v) Ponceau S (sodium salt) (Sigma, MO, USA)
- 30% (w/v) Trichloroacetic acid CCl_3COOH
- 30% (w/v) 5-sulfosalicylic acid $C_7H_6O_6S \cdot 2H_2O$ (Sigma, MO, USA).

The solution was dissolved in water to a final concentration of 1x before use.

Puromycin

A 2mg/ml stock was made by dissolving puromycin (Sigma) into distilled water. The solution was used at a final concentration of $1\mu\text{g/ml}$

Propidium Iodide Solution

A stock solution of 1 mg/ml (20x) Propidium iodide $C_{27}H_{34}N_4I_2$ (Sigma) was made up in sterile distilled water. This was stored in single-use aliquots in the dark at $-20\text{ }^{\circ}\text{C}$. The PI solution was made up in 1xPBS as follows

- 50mg/ml RNA'seA (Boeringer Mannheim)
- 25 mg/ml Propidium iodide

Protein G Sepharose

Stored in 20% ethanol at 4°C (Pharmacia Biotech)

Puromycin

A 2.5 mg/ml puromycin (Sigma, UK) stock solution was prepared in Millipore water and filter-sterilised and frozen in aliquots, it was used at 2.5 mg/ml.

Qiagen Solution-1-Resuspension solution for plasmid preparations

- 50 mM Tris-HCl pH 8.0
- 10 mM EDTA pH 8.0
- $100\mu\text{g/ml}$ RNase A (stored at $4\text{ }^{\circ}\text{C}$ after addition of RNase)

Qiagen Solution-2-Lysis solution for plasmid preparations

200 mM NaOH

1% (w/v) SDS

Qiagen Solution-3-Neutralizing solution for plasmid preparations

3 M CH₃COOH, pH 5.5

Qiagen Elution Buffer

1.25M NaCl

50 mM Tris pH8.5

15% Isopropanol

Qiagen Equilibration Buffer

750 mM NaCl

50 mM MOPS pH 7.0

15 % Isopropanol

0.15% Triton® X-100

Qiagen Wash Buffer

1 M NaCl

50 mM MOPS pH 7.0

15% Isopropanol

RIPA (radioimmunoprecipitation) Lysis Buffer

150 mM NaCl

1% (v/v) NP40

0.5% (v/v) Sodium deoxycholate (DOC)

0.1% (w/v) SDS

50 mM Tris-HCl (pH 8.0)

2 mM PMSF (Sigma) (*added fresh each time*)

1/20 COmplete™ Protein Inhibitor Cocktail

Ras IP buffer

20mM	Trish-HCl pH 7.5
1mM	EDTA
1000mM	KCl
5mM	MgCl ₂
10% v/v	glycerol
1% v/v	Triton X-100
0.05% v/v	2-Mercaptoethanol

On the day add

5mM	NaF
0.2mM	Na ₃ VO ₄
5µg/ml	aprotinin
5µg/ml	leupeptin
1mM	Benzamidine
1mM	p-aminoethyl-benzyne sulfonyl fluoride

Ribonuclease A (RNase A)

50 mg of ribonuclease A (Sigma) was dissolved in 1 ml of 10mM Tris-HCl pH7.5 and 15mM NaCl to make a 10 mg/ml stock solution which was stored in single-use aliquots at –20 °C. (Boiling to remove DNase was not recommended by the manufacturer).

Reporter Lysis 5x Buffer

This lysis buffer was purchased from Promega, UK as part of the luciferase assay kit. To use it was it was diluted with distilled water to make a 1x solution.

SDS Solution

A 10% (w/v) solution of sodium dodecyl sulphate (SDS) was dissolved in water and stored at room temperature.

6X SDS-PAGE sample buffer

750mM	0.5 M Tris pH 6.8 (33.3ml in 50 ml total)
30%	Glycerol (15 ml in 50 ml total)
6%	10% SDS (3g in 50ml total)
0.03% (w/v)	Bromophenol blue
60mM	2-mercaptoethanol (210 μ l in 50ml total) <i>added just before use</i>

10x SDS-PAGE Running Buffer

720 g	Glycine
150 g	Tris
50 g	SDS

Made up to 5 litres with distilled water.

10x SDS-PAGE Transfer Buffer

725 g	Glycine
145 g	Tris

Made up to 5 litres with distilled water.

Stripping Buffer

62.5 mM	15.5 ml of 1 M Tris-HCl, pH 6.7
100 mM	1.75 ml of β -mercaptoethanol
2%	5 g SDS

Make up to 250 ml in distilled water.

Stripping Buffer, commercial

Provided by Chemicon Internaional, USA

50x TAE (Tris-Acetate-EDTA) buffer

242 g	Tris base
57.1 ml	Glacial acetic acid
100 ml	0.5 M EDTA, pH 8.0

Make up to 1 litre with distilled water and adjust pH to about 8.5. Use at 1x concentration. For making an agarose gel, 2 µl of 10 mg/ml Ethidium bromide solution per 100 ml was added to the 1 x TAE.

Tetracycline

A 5mg/ml stock was made by dissolving tetracycline (Sigma) in 100% ethanol. The solution was used at a final concentration of 2µg/ml.

Thrombin buffer

50mM	Tris-HCl, pH 7.5
2.5mM	CaCl ₂
100mM	NaCl
5mM	MgCl ₂
Immediately before use add	
1mM	DTT

Tris/Sucrose buffer

10mM	Tris pH 7.4
0.5mM	EDTA
0.3M	Sucrose

Tris/Sucrose buffer with NaCl

10mM	Tris pH 7.4
0.5mM	EDTA
0.3M	Sucrose
0.5M	NaCl

Tris Stock solutions

Tris base was dissolved in water to provide 0.5 M, 1 M and 1.5 M solutions which were pH adjusted with concentrated HCl.

10x Tris Buffered Saline Tween (TBS-T)

121 g Tris base

36.53 g NaCl

250 ml Tween-20 (Sigma)

Adjust pH to 7.6 with about 60 ml HCl in a total volume of 5 litres. Used at 1x concentration.

Triton® X-100

A 20% (v/v) stock solution in PBS was made and stored at room temperature

Trypsin solution

50 mM ammonium bicarbonate (500 µl of 0.1 M)

25 µl 316 detergent (2%-0.05%)

475 µl MilliQ

TE Buffer

10 mM 1.0 ml of 1 M Tris-HCl pH 8.0 (sterile)

1 mM 0.2 ml of 0.5 M EDTA, pH8.0 (sterile)

Make up to 100 ml of sterile distilled water.

Trypan Blue (Sigma)

Trypan blue solution (0.4%)

In 0.81% Sodium Chloride and 0.06% Potassium phosphate

10x TTBS

0.5M NaCl

20 mM Tri-HCl, pH 8.0

0.1% (v/v) Tween-20

0.01% NaN₃

2x TYE Medium (per litre)

1.6% (w/v) 16g Bacto-tryptone (Difco, USA)
1% (w/v) 10g Bacto-yeast extract (Difco, USA)
85 mM 5g NaCl

The pH was adjusted to 7.4 and the solution autoclaved. This solution was prepared by the Ludwig washroom team.

TYE Plates (per litre)

1% (w/v) 10g Bactotryptone (Difco, USA)
0.5% (w/v) 5g Bacto-yeast extract (Difco, USA)
140 mM 8g NaCl
1.5% (w/v) 15g Agar (Difco, USA)

The pH was adjusted to 7.4 and the solution autoclaved and poured into tissue culture plates and stored at 4 °C. This solution was prepared by the Ludwig washroom team.

TYE Ampicilin Plates (per litre)

1% (w/v) 10g Bactotryptone (Difco, USA)
0.5% (w/v) 5g Bacto-yeast extract (Difco, USA)
140 mM 8g NaCl
1.5% (w/v) 15g Agar (Difco, USA)
50 µg/ml Ampicilin

The solution was adjusted to pH7.4 and autoclaved. At 50°C, ampicillin was added to a final concentration of 75 µg/ml and the plates poured. The solidified plates were stored at 4°C not more than for one month. This was prepared by the Ludwig washroom team.

UO126

The MEK inhibitor UO126 was ordered from Calbiochem. 1mg of powder was dissolved in DMSO to give a stock concentration of 50mM. The solution was used immediately at a working solution of 20 µM.

Water

Nanopure water (Type I) generated from the MilliQ water system was used for all procedures.

øX174-HaeIII DNA Marker

The øX174 DNA was digested with HaeIII to generate 11 fragments ranging in size from 72 to 1,353 bp This marker, supplied as a 1 mg/ml solution, was used as a size standard for agarose gel electrophoresis.

2.1.2 SDS-PAGE gels

	Resolving Gels				Stacking
	6%	8%	10%	12%	4 %
Acryl/Bis	2 ml	2.7 ml	3.3 ml	4.0 ml	1.3 ml
1.5 M Tris-HCl pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml	--
1.0 M Tris-HCl pH 6.8	--	--	--	--	2.5 ml
10% SDS	100 µl	100 µl	100 µl	100 µl	100 µl
10% APS	100 µl	100 µl	100 µl	100 µl	50 µl
TEMED	10 µl	8 µl	5 µl	5 µl	10 µl
Distilled Water	5.3 ml	4.6 ml	4.0 ml	3.3 ml	6.1 ml
Total volume	10 ml	10 ml	10 ml	10 ml	10 ml

All resolving and stacking gels were prepared using 30% acrylamide/bis-acrylamide (Acryl/Bis) 29:1 (NBL, UK or BioRad, UK). Values given are per 10 ml of gel required. Abbreviations: Ammonium Persulphate (APS); N,N,N',N',-tetramethylethylenediamine (TEMED), Tris (Tris(hydroxymethyl) aminomethane), sodium dodecyl sulphate (SDS).

2.1.3. Antibodies

Antigen	Antibody Name	Species	Source
ASPP1	YX.7	Mouse mAb	Serum
ASPP1	LX054.2	Mouse mAb	Serum
ASPP1	LX054.1	Mouse mAb	Serum
ASPP1	ASPP1.88	Rabbit pAb	Serum
ASPP2	DX54.10	Mouse mAb	Hybridoma
ASPP2	BP77	Rabbit pAb	Serum
CD-20	347673	Mouse-FITC	Becton-Dickson (CA, USA)
GST	Z-5, sc-459	Rabbit pAb	Santa Cruz (CA, USA)
GST	B-14, sc-138	Mouse mAb	Santa Cruz (CA, USA)
HA	HA.11	Mouse mAb	Convance
H/K-Ras	259	Rat mAb	Santa Cruz (CA, USA)
H/K-Ras	238	Rat mAb	Santa Cruz (CA, USA)
H-Ras	R02102	Mouse mAb	BD Transuction Laboratories (USA)
iASPP	iASPP	Mouse mAb	Serum
K-Ras	F234, sc-30	Mouse mAb	Santa Cruz (CA, USA)
MAPK1/2	K-23, sc-94	Rabbit pAb	Santa Cruz (CA, USA)
Mouse IgG	Mouse IgG	Mouse serum	Sigma
Mouse-IgG	715-095-151	Donkey-FITC	Jackson ImmunoResearch
Mouse-IgG	715-035-151	Donkey-HRP	Jackson ImmunoResearch
Mouse-IgG	415-005-100	Rat	Jackson ImmunoResearch

Mouse-IgG	415-005-166	Rat	Jackson ImmunoResearch
p53	DO-1	Mouse mAb	Hybridoma
p53	DO-13	Mouse mAb	Serum
PCNA	PC-10	Mouse mAb	Hybridoma
Phospho-MAPK	9106	Mouse mAb	Cell Signaling Technology
Raf-1	C-12, sc-13	Rabbit pAb	Santa Cruz (CA, USA)
Raf-1	540	Mouse mAb	Anogen (Ontario, Canada)
V5	46-0705	Mouse mAb	Invitrogen
Rat-IgG	712-095-153	Donkey-FITC	Jackson ImmunoResearch

Abbreviations – monoclonal antibody (mAb), polyclonal antibody (pAb), horse-radish peroxidase (HRP), Fluorescein isothiocyanate (FITC), Tetramethylrhodamine isothiocyanate (TRITC).

Protein Molecular weight markers

The Rainbow™ coloured protein molecular weight markers (14490-220000 kDa) were purchased from Amersham Life Science (UK). The prestained protein markers broadrange (#P7708S) were purchased from New England Biolabs

2.1.4. Plasmids

Plasmid Name	Relevant Information	Source / Reference:
ASPP1 fragmen6-PCDNA	Human ASPP1 bases 1074-3386 cloned into PCDNA3	Susana Llanos
ASPP1 fragmen8-PCDNA	Human ASPP1 bases 2082-3386 cloned into PCDNA3	Susana Llanos
ASPP1 fragment (1-308)	Human ASPP1 bases 1-308 cloned into pCRT73	Alan Renton
ASPP1 fragment1-PCDNA	Human ASPP1 bases 1-1074 cloned into PCDNA3	Susana Llanos
ASPP1 fragment2-PCDNA	Human ASPP1 bases 1-2802 cloned into PCDNA3	Susana Llanos
ASPP1 fragment4-PCDNA	Human ASPP1 bases 1074-2802 cloned into PCDNA3	Susana Llanos
ASPP2 fragment A-PCDNA	Human ASPP2 bases 1-1080 cloned into PCDNA3	Susana Llanos
ASPP2 fragment B-PCDNA	Human ASPP2 bases 360-925 cloned into PCDNA3	Susana Llanos
ASPP2 fragment C-PCDNA3	Human ASPP2 bases 925-1128 cloned into PCDNA3	Susana Llanos
ASPP2 Mut1 (S698A)-PCDNA3	Human ASPP2 containing a point mutation at amino acid 698 from serine to alanine, cloned into PDNA3	Nadia Godin-Heymann
ASPP2 Mut2 (S736A;S737A)-	Human ASPP2 containing two point mutations at amino acid 636	Nadia Godin-Heymann

PCDNA3	and 737 from serine to alanine, cloned into PDNA3	
ASPP2 Mut3 (S827A)-PCDNA3	Human ASPP2 containing a point mutation at amino acid 827 from serine to alanine, cloned into PCDNA3	Nadia Godin-Heymann
CMV-Bam-Neo	Empty vector, Neo-resistant, CMV	Previously obtained by Xin Lu
EXV-H-Ras	Wild type H-Ras cloned in an EXV vector	Chris Marshall
EXV-H-Ras N17	Dominant negative H-Ras cloned in an EXV vector	Chris Marshall
p21-luc	p53-responsive p21 promoter linked to luciferase reporter	Bert Vogelstein
pcDNA3 53BP2	Human 53BP2 driven by the CMV promoter, has a T7 promoter	S.Fields (recloned by Shan Zhong)
pcDNA3 ASPP1	ASPP1 driven by the CMV promoter, has a T7 promoter	Shan Zhong
pcDNA3 ASPP2	ASPP2 driven by the CMV promoter, has a T7 promoter	Shan Zhong
pCDNA3 E6	Adenoviral E6 driven by the CMV promoter and has the T7 promoter	Tim Crook (recloned by Isabelle Campargue)
pcDNA3 -iASPP	Human iASPP driven by the CMV promoter, has a T7 promoter	Isabelle Campargue
pCDNA3.1- anti-sense ASPP2	Human ASPP2 in reverse orientation driven by the CMV promoter and has the T7 promoter	Giuseppe Trigiante
PCDNA3.1/V5- His-TOPO	Empty vector containing CMV and 7 promoter sites	Invitrogen

pCMV ASPP2	ASPP2 driven by the CMV promoter	L.Naumovski
pCMV Bbp	Human 53BP2 driven by the CMV promoter	L.Naumovski
pCMV E2F1	Human E2F1, driven by the CMV promoter	Kristian Helin (1993b)
pEFm.6	Empty vector containing a EF 1a promoter.	Richard Marais
pEFm.6 – Raf CX	Activated Raf1 cloned into the pEFm.6 vector	Richard Marais
pEFm.6 HA-H-Ras V12	Activated H-Ras with an HA tag	Richard Marais
pEFm.6 HA-K-Ras V12	Activated K-Ras with an HA tag	Richard Marais
pEFm.6-H-Ras V12	Activated H-Ras with a point mutation at codon 12 changing the glycine to a valine, cloned into the pEFm.6 vector	Richard Marais
PGEX-2TK-53BP2	ASPP2 (693-1128) tagged to GST	Isabelle Campargue
PGL3 <i>Bax</i> -luc	<i>bax</i> promoter linked to luciferase reporter	Moshe Oren (original from Miyashita et al., 1994b)
PGL3 <i>mdm2</i> -luc	p53-responsive <i>mdm2</i> (P2) promoter (intron 1) linked to luciferase reporter	Karen Vousden (original from Zauberman et al., 1995)
PIG3-10mer-luc	P53-responsive PIG3 promoter containing 10 repeats of p53 binding sites linked to luciferase reporter	Matthias Dobelstein

PIG3-15mer-luc	P53-responsive PIG3 promoter containing 15 repeats of p53 binding sites linked to luciferase reporter	Matthias Dobelstein
PIG3-17mer-luc	P53-responsive PIG3 promoter containing 17 repeats of p53 binding sites linked to luciferase reporter	Matthias Dobelstein
PIG3-luc	p53-responsive PIG3 promoter linked to luciferase reporter	Bert Vogelstein
SN3 (CMV p53)	Human p53 driven by the CMV promoter	Bert Vogelstein (Baker et al., 1990)
T7-p53	Human p53, driven by the T7 promoter	Previously obtained by Xin Lu (re-cloned from the original SN3)

All plasmids constructed by our laboratory were sequenced to confirm their identity. All cDNA encode human wild-type proteins unless otherwise stated.

2.1.5. Cell lines

Name	Tissue Type/Origin
SAOS-2	Human osteosarcoma; p53 null, truncated Rb
H1299	Human lung carcinoma; p53-null
MCF7	Human Breast, wild type p53

U2OS	Human osteosarcoma, wild type p53
MEFs	Mouse embryo fibroblasts prepared from ASPP2 heterozygous crosses

All cells except for MEFs were obtained from American Type Culture Collection (ATCC) and stored in liquid nitrogen until required.

Constructed cell lines

Name	Description
Tet off U2OS	Human osteosarcoma, wild type p53, stably transfected with pTet-off plasmid
ASPP2 –inducible clonw 25	Human osteosarcoma, wild type p53, stably transfected with pTet-off and ASPP2 plasmids. Provided by Yardená Samuels.

Isolating Mouse Embryo Fibroblasts (MEFs)

The mother mouse was sacrificed by the technicians in the animal house. She was brought to a tissue culture hood where she was attached securely with pins at her hands and feet with her stomach facing upwards and she was sprayed with ethanol profusely. The womb/stomach was cut open with scissors and the two placentas containing the embryos were pulled out and put in a 10 cm sterile dish with cold sterile PBS. With the help of two tweezers each embryo was dissected out of its placenta bag. The head of each embryo was removed and put in fixing solution. All red organs were removed and put in an eppendorf tube with lysate solution. The remainings of each embryo was put in a clean 10cm dish and was cut up with a scalpel until it was a monolayer. 2 ml of trypsin was added and the embryonic cells were put in the incubator for 30-45 minutes. 12 ml of growth medium containing foetal calf serum was added to the trypsinized cells and resuspended gently. The resuspended cells were put in a 15 ml falcon tube. The supernatant was added to a 225cm flask (Corning Incorporated) and left overnight in the 37 C incubator. Once confluent the mouse embryo fibroblasts (MEFs) were split 1:3

and then frozen down. The red organs were used to determine the genotype of each embryo by PCR.

2.2. Methods

2.2.1. Tissue Culture

Basic Media

RPMI 1640 and Dulbecco's Modified Eagle's Medium (DMEM) were from Gibco-BRL, UK and stored at 4 °C.

Media supplements

Foetal calf serum (FCS) was purchased from PAA Laboratories and tested for its ability to support growth of various cell lines. It was heated inactivated for 30 minutes at 55 °C and stored at -20 °C in 50 ml aliquots.

L-Glutamine was purchased from Gibco-BRL at a 200mM concentration stored at -20 °C and used at a final concentration of 2 mM.

Penicillin / Streptomycin were purchased from Gibco-BRL at 1000,000 unit/ml stored at -20 °C and used at a final concentration of 200 units/ml.

Maintaining cell lines

All the cell lines are cultured in the Complete Medium (DMEM or RPMI 1640) supplemented L-Glutamine, penicillin / streptomycin and 10% (v/v) of foetal calf serum) in the flasks or dishes (Falcon) maintained in the Heraecus incubator at 37 °C in the presence of 10% CO₂. Medium was changed every 3-5 days depending on the cell lines. On reaching confluence, the cell were washed once with 1X PBS and incubated with 2-4 ml pre-warmed Trypsin-EDTA (Gibco, BRL) at 37 °C until the cells detached from the flasks or dishes. Trypsin was inhibited by addition of an appropriate volume of fresh growth medium and this culture was then seeded on to fresh flasks or dishes at the desired density.

Freezing / Thawing of cells

Cells were seeded the day prior to freezing at a density such that they would be 70% confluent on the day of freezing. Cells from the growing culture were detached by trypsinising with 0.5 ml Trypsin-EDTA then resuspended in 2 ml of freezing medium (10% v/v of DMSO , 90% v/v of FCS in DMEM) to the dilution around $1-5 \times 10^7$ cells

per ml. 1 ml aliquots of the cells suspensions were transferred into 2ml freezing ampoule (Corning). The vials then labelled and cooled at the rate of 1 °C per minute in a Nalgen Cryo 1 °C freezing container or in a tissue-insulated polystyrene box when placed in a -80 °C freezer (New Brunswick Scientific) for at least 24 hr before being transferred to liquid nitrogen tank for long term storage.

To thaw cells from liquid nitrogen stock, vials were placed in the 37 °C water bath for 2 minutes and then transferred to a 6cm or 10cm dish with the appropriate pre-warmed fresh growth medium and kept in the 37 °C incubator overnight for recovering.

2.2.1. DNA Techniques

Bacterial strains and culture

The *Escherichia coli* strains DH5α or BL21 were used as host strains for plasmid DNA. Bacteria were cultured at 37 °C in 2X TY broth with appropriate antibiotic (such as 100 µg/ml Ampicillin) according to resistance gene carried by the plasmid DNA for selection of transformed bacterium. Both the broth and the bacterial plates were supplied by the Laboratory services.

Preparing competent bacterial cells

A single colony of the required *E.coli* strain was picked from 2x TY plate and inoculated in 3 ml 2x TY without any antibiotics and cultured at 37 °C with shaking overnight. 1 ml of this culture was use to inoculate a 500 ml 2x TY flask without antibiotics and incubated at 37 °C with shaking for about 2-3 hr, the OD at 600 nm was measured at appropriate intervals till reaches 0.95. The cells then were pelleted for 5 minutes at 490 g (IEC PR-7000) at 4 °C and washed with 10 ml of sterile ice-cold solution containing 80 mM CaCl₂ and 50 mM MgCl₂. The bacteria were pelleted again then resuspended in 10 ml sterile cold 0.1 M CaCl₂ (to a final concentration of 5 x 10⁹ cells /ml) and incubated on ice for 20 minutes. After the addition of sterile-filtered 50% glycerol, the bacteria cells were aliquoted into sterile Eppendorf tubes and snap frozen on dry ice/ethanol. The tubes were then stored at -70 °C. Freezing and thawing the same aliquot was avoided.

Transformation

The competent cells were thawed on ice. The desired plasmid DNA (100 ng) was added to the vial of competent cells, and this was incubated on ice for 30 minutes. The bacteria were subjected to heat shock for 52 seconds at 42 °C in a water bath, then incubated on ice for another 2 minute. 500µl of 2x TY without antibiotics was added to the Eppendorf tube and left to shake at 37 °C for 30 minutes to 1 hr before plating on 2x TY plates with an appropriate antibiotic. Plates were then incubated at 37 °C overnight.

Small scale preparation of plasmid DNA (mini-prep)

A single bacterial colony was inoculated in 3 ml of 2X TY/antibiotic medium in a sterile test tube. The medium was shaken at 37°C for 16 hours then 2 ml was removed and centrifuged at 3000 rpm, at 4°C for 2 minutes (eppendorf 5417R centrifuge/F-45-24-11 rotor). The supernatant was discarded into bleach and the pellet resuspended in 300 µl of Qiagen Solution 1. Subsequently 300 µl of Solution 2 was gently mixed in by inverting a few times and left for 5 minutes at room temperature. 300 µl of Qiagen Solution 3 was added on ice, mixed and left for 5 minutes. The mixture was then centrifuged (eppendorf 5417R centrifuge/F-45-24-11 rotor) at 14000 rpm at 4°C for 10 minutes and the supernatant removed to a fresh eppendorf and mixed with 650 µl of isopropanol. The solution was inverted a few times to mix and then was centrifuged at 14000 rpm for 10 minutes at 4°C. The supernatant was discarded carefully so as not to disrupt the pellet. 500 µl of 70% ethanol was used to wash the pellet twice. The DNA was dried in a DNA speed vaccum (Savant DNA 10) at high speed and high temperature for 10 minutes. 50µl of TE buffer was added to resuspend the DNA pellet and the concentration measured by OD260 or gel analysis.

Large scale preparation of plasmid DNA (maxi-prep)

A single bacterial colony was inoculated in 5 ml of 2X TY/antibiotic medium in a sterile test tube, and shaken at 37°C for 4 hours. The resulting bacterial suspension was used to inoculate a 250 ml flask of 2X TY/antibiotic medium and shaken for a further 16 hours at 37°C. The cells were spinned down at 3000g for 10 minutes at 4°C (Sorvall RC 5C Plus, rotor SLA-3000). The large scale DNA preparation was carried out according to Qiagen Qiafilter Maxi DNA kit protocol.

Phenol:chloroform extraction

Phenol saturated in TE was mixed in a 1:1 ratio with chloroform. The DNA solution to be purified was added to the phenol:chloroform solution at a 1:1 ratio and vortexed. The aqueous phase containing the DNA was separated from organic phase containing impurities by centrifugation at 14000 rpm (Eppendorf Centrifuge 5415C) for 2 minutes at room temperature and transferred to fresh tubes. This procedure was carried out twice with phenol:chloroform and once with chloroform alone in the same way. DNA was precipitated from the aqueous phase solution by addition of 1/10 volume of 3 M sodium acetate to a final concentration of 0.3 M followed by 70% of the total volume of isopropanol. The DNA was pelleted by centrifugation at 14 000 rpm for 30 minutes at 4°C and washed twice with 70% of ethanol and air-dried before resuspension in an appropriate buffer (TE, pH 8.0).

Concentration determination of DNA

DNA preparations were diluted 1:500 in double distilled H₂O (ddH₂O) and the optical density (OD) at 260 nm was measured against a ddH₂O blank in a spectrophotometer (Perkin Elmer). If a DNA preparation is pure, the ratio of OD 260/OD 280 should be around 1.8. In some cases, quantity and purity was also checked by running it on a agarose gel against a DNA marker of known quantity

Agarose gel electrophoresis

DNA samples were mixed with 5xDNA loading buffer to a final concentration of 1x before being resolved on appropriate percentage of agarose gel according to the size of the DNA bands to be visualised. The gels were made with TAE buffer containing ethidium bromide and run at 60-80 V with DNA makers. The ethidium bromide-incorporated DNA bands were then visualised under UV-irradiation.

Cloning

DNA was subcloned following the standard method as described (Sambrook et al., 1989). DNA was digested with suitable restriction enzymes and resolved on a gel. The required band was excised and purified using the Qiagen PCR purification kit. The DNA was then ligated into the linearised, dephosphorylated and purified vectors.

Restriction digestion of DNA

1 μg of DNA was incubated with the appropriate amount of enzyme (5 U/ 1 μg of plasmid DNA) with the respective buffer in a reaction volume of 25 μl and incubated at 37 °C for 3 hours to overnight. Larger quantities of DNA were digested in scaled up procedures.

Dephosphorylation

Linearised vector DNA (2.5 μg) was treated with 5U / μg of plasmid DNA of calf intestinal alkaline phosphatase (CIAP) in the appropriate buffer in a total reaction volume of 50 μl . The reaction was incubated for 2 hours at 37 °C to remove 5' phosphate ends to prevent self-re-ligation.

Ligation

Ligations were set up with approximately 5 fold excess of insert DNA to vector, using T4 DNA ligase (400 U / 1 μg recovered vector). Reactions were incubated at room temperature for 2 hours or overnight at 15 °C, then used to transform highly-competent bacteria cells (DH5 α strain) which were then plated out on media with the selectable antibiotic.

RNAi cloning

pSUPPRESSOR vector

All inserts in the pSUPPRESSOR vector start with the sequence TCGAG followed by a gene-specific insert 19-nucleotide sequence which is separated by a 9-nucleotide non-complementary spacer (ttcaagaga) from the reverse complement of the same 19-nucleotide sequence. All inserts end with a string of six T nucleotides.

In the case of H-Ras-pSUPPRESSOR, the 19 nucleotides insert corresponds to nucleotides 299-315 downstream of the transcription start site.

The H-Ras forward primer: TCGAG TC AAA CGG GTG AAG GAC TC ttcaagaga
GA GTC CTT CAC CCG TTT GA tttttt

The H-Ras reverse primer: CTAG aaaaa TC AAA CGG GTG AAG GAC TC
tctcttgaa GA GTC CTT CAC CCG TTT GA C

In the case of K-Ras-pSUPPRESSOR, the 19-nucleotide insert corresponds to nucleotides 25-43 downstream of the transcription start site.

The K-Ras forward primer: TCGAG GTT GGA GCT GGT GGC GTA G ttcaagaga
CTA CGC CAC CAG CTC CAA C tttt

The K-Ras reverse primer: CTAG aaaaa G TTG GAG CTG GTG GCG TAG
tctcttgaa C TAC GCC ACC AGC TCC AAC C

pSUPER vector

The same inserts were used as for pSUPPRESSOR with the minor difference that the sequences have slightly different tags for the forward and reverse primers. The forward primer has the 5' tag GATCCCC before the appropriate gene insert, followed by the same non-complementary sequence (ttcaagaga) before the reverse complement of the sequence and ends with TTTT GG AAA. The reverse primer has the tag AGC TTTT CC AAAAA, followed by the appropriate gene insert, including the non-complementary sequence, and ends with GGG.

Site-directed mutagenesis

Two complimentary oligonucleotides containing the desired mutation flanked by unmodified nucleotide sequence were synthesised. The mutants were made following the QuickChange® Site-Directed Mutagenesis Kit protocol (Stratagene). A series of sample reactions using various concentrations of dsDNA template ranging from 0-50ng was performed:

<u>Reagent</u>	<u>Concentration</u>
10x reaction buffer	5 µl
dsDNA template (53BP2)	0-50ng
Primer 1	125 ng
Primer 2	125 ng
DNTP mix	1 µl
ddH ₂ O	final volume of 50 µl
<i>Pfu turbo</i> DNA polymerase	1 µl (2.5 Units/µl)

The primers used to make ASPP1 mutant (S671A):

Forward: CTG CCA CGG CCA CTC GCC CCC ACC AAG CTC ACG

Reverse: CGT GAG CTT GGT GGG GGC GAG TGG CCG TGG CAG

The primers used to make ASPP1 mutant (S746A):

Forward: ACC CCT TTC TAC CAG CCC GCC CCC TCC CAG GAC TCC

Reverse: GAA GTC CTG GGA GGG GGC GGG CTG GTA GAA AGG GGT

For ASPP1 mutants, the primers described above were set up in a PCR reaction:

<u>Temperature</u>	<u>Time</u>
<u>1 cycle</u>	
94°C	2 minutes
<u>2-3 cycles</u>	
94°C	1 minute
35°C	1 minute
68°C	10 minutes
<u>16-18 cycles</u>	
94°C	1 minute
58°C	1 minute
68°C	10 minutes
<u>1 cycle</u>	
68°C	20 minutes
4°C	∞

The primers used to make ASPP2 mutant (S698A):

Forward: CCT CGG CCA CTC GCC CCA ACT AAA TTA CTG CC

Reverse: GG CAG TAA TTT AGT TGG GGC GAG TGG CCG AGG

The primers used to make ASPP2 mutant (S736A, S737A):

Forward: GG CCT CTA AAG AAA CGT GCG GCT ATT ACA GAG CCA GAG
GGT CC

Reverse: GG ACC CTC TGG CTC TGT AAT AGC CGC ACG TTT CTT TAG AGG
CC

The primers used to make ASPP2 mutant (S827A):

Forward: CT GAC ATG CCA GCT CCT GCT CCA GGC CTT GAT TAT GAG CC

Reverse: GG CTC ATA ATC AAG GCC TGG AGC AGG AGC TGG CAT GTC AC

For ASPP2 mutants, the primers described above were set up in a PCR reaction (note that the mutants were not made in full length ASPP2 but in ASPP2 fragment (694-1128)).

<u>Temperature</u>	<u>Time</u>
<u>1 cycle</u>	
95°C	30 seconds
<u>12-18 cycles</u>	
95°C	30 seconds
55°C	1 minute
68°C	14 minutes
<u>1 cycle</u>	
68°C	7 minutes
4°C	∞

The efficiency of the PCR reaction was analysed by gel electrophoresis and 1 µl of Dpn I restriction enzyme (10 units/µl) was added directly to each successful PCR reaction. The reaction mixtures were incubated for 1 hour at 37°C to digest the parental supercoiled dsDNA.

An aliquot of the digested PCR product was used to transform super-competent bugs and maxi-prep the colonies containing the mutant DNA.

General conditions for PCR Cycles

<u>Temperature</u>	<u>Time</u>	
<u>1 cycle</u>		
94 °C	7 min	
<u>35 cycles</u>		
94 °C	30 seconds	
52 °C	40 seconds	
72 °C	2 mins/Kb	
<u>1 cycle</u>		
72 °C	10 min	-
4 °C	∞	

PCR products were resolved on a 1.5% agarose/TAE gel at 80V for 1-2 hrs.

2.2.3. RNA Manipulations

Total RNA was isolated using RNAzol™B. Pellets of 10^7 cells were resuspended in 2ml of RNAzol™B (which contains guanidium thiocyanate for cell lysis). 0.2ml of chloroform was added and the sample was vortexed before incubation on ice for 5min. After centrifugation at 12000g (4°C, 30min), an equal volume of isopropanol was added to aid precipitation of RNA. RNA quality was checked by electrophoresis on a 1% agarose/TAE gel. RNA was used in a cDNA synthesis reaction using the ProSTAR Ultra HF RT-PCR system (Stratagene). A 10ml reaction consisted of the following reagents:

<u>Reagent</u>	<u>Concentration</u>
10xRT buffer	10mM Tris-HCl (pH8.8), 50mM KCl, % Triton® X-100
dNTP's	1mM each dNTP
RNA	0.05mg/ ml
Oligo(dT) ₁₅ Primer	0.6ml
RNase-free water	to 10 ml

These were incubated at 65°C for 5min. The reaction was then cooled at RT to allow the primers to anneal to the RNA. The following reagents are then added:

rRNasin Ribonuclease	0.4 unit/ml
inhibitor	
MMLV-RT	0.2 units/ml

The tubes were then placed at 37°C and incubated for 30min. Each cDNA synthesis reaction was carried out in duplicate, with AMV reverse transcriptase omitted from one reaction as a control for genomic DNA contamination. cDNA was then stored at -20 °C until use.

For the PCR reactions, 2ml of cDNA was used. All PCR reactions were carried out in a total volume of 25ml with reagents (Stratagene,UK) at the following final concentrations:

<u>Reagent</u>	<u>Concentration</u>
Primers	2.2pmol
cDNA	2ml
MgCl ₂	1 mM
dNTPs	200 mM
10x <i>Pfx</i> amplification buffer	2.5 ml
Taq polymerase	0.1 ml (2.5 units/ml)
H ₂ O	up to 25 ml

The PCR reactions were carried out using a Perkin-Elmer DNA thermal Cycler-480 was used to achieve denaturation, annealing and synthesis.

2.2.4. Protein Manipulations

Affinity purification of phospho-specific antibody

Using the peptide CPAPSpPGLDY (representing residues 824-832) with the serine phosphorylated as an immunogen, the rabbit polyclonal antibody S-4 which specifically recognizes phosphorylated ASPP2 at amino acid 827 was raised.

0.5 g of epoxy-activated-sepharose-6B (Amersham Pharmacia Biotech) was washed with 50 ml of water 7 times (spinned at 3000 rpm, 5 mins in Beckman centrifuge) and 3 times in 50 ml NaHCO_3 . The bed volume (approximately 1ml) was resuspended 1:1 in NaHCO_3 . 10 mg of peptide was then resuspended in 0.5 ml of 0.1 M NaHCO_3 , pH 9.0. The peptide was combined with the sepharose and left to rotate at 37 °C for 20 hours. The sepharose column was washed with 0.1 M NaHCO_3 , pH 9.0 twice and was subsequently blocked with 5 ml of 0.1 M mercaptoethanol (diluted in water) for 2 hours at room temperature on a roller. The sepharose column was washed with three cycles of 10 ml 0.2 M glycine, pH 2.8 followed by 10 ml of 0.1 M NaHCO_3 + 0.5 M NaCl. 10 ml of serum from the final bleed was added to 1ml 10x TTBS and the serum was then clarified by centrifugation and filtered through 0.45 μm filter. The antibody was absorbed by transferring the affinity resin to the tube (15 ml falcon tube) with the serum and left to rotate overnight at 4°C. The antibody and affinity column mixture was added to a poly-prep column (Bio-Rad) and the run-through serum was stored at 4°C. The column was washed extensively with 1x TTBS until the flow-through had an $\text{OD}_{280\text{nm}} < 0.01$ (approximately 200 ml). The antibody was then eluted with 5 ml of 0.2M glycine (pH 2.8) and neutralized with Tris-HCl (pH 8.0). Fractions of approximately 500 μl of the eluted antibody were collected in 1.5 ml eppendorf tubes containing between 5-40 μl of Tris-HCl (pH 8.0) (the optimum volume of Tris-HCl (pH 8.0) to neutralize the glycine was measured just prior to elution). The first two fractions were pooled together, as were the third and fourth fractions. The pooled fractions were then dialyzed overnight in 5 liters of PBS at 4°C. NaN_3 was added to the antibodies and they were stored at 4°C. The affinity column was washed extensively with 0.2 M glycine, pH 2.8 and then washed extensively with 1x TTBS, after which it was stored in TTBS and NaN_3 at 4°C.

Sample preparation

Cells grown in monolayers were washed three times with 1X PBS and lysed in RIPA lysis buffer, luciferase lysis buffer (Promega) or Ras IP buffer as indicated (150-250 μ l per 10 cm dish). The cells were scraped with a sterile disposable cell scraper (Greiner) and transferred to an eppendorf tube and centrifuged at 14000 rpm, 4°C for 10 minutes (eppendorf 5417R). The resulting lysate was removed to a fresh eppendorf tube and the cell debris discarded. The lysate was removed for further analysis.

Protein concentration determination

The protein concentrations of cell extracts were determined using BioRad protein assay reagent system. 1 μ l of cell lysate was mixed with 150 μ l of 1x BioRad assay reagent and then measured at 595 nm in the spectrophotometer (Anthos Labtech instrument). All samples were measured in duplicates and the absorbance was compared against the standard curve made at the same time from known concentrations of bovine serum albumin (BSA; Sigma, UK) in the same solutions using the same method.

Preparation of SDS-PAGE gels

All plates were washed with water and detergent, dried and assembled in the casting trays (Pharmacia BioTech, UK). The acrylamide content of the gels varied between 8%-12% depending on the size of the protein of interest. The acrylamide gels were overlaid with 70% isopropanol solution and left to polymerise. After polymerisation, the isopropanol was removed and a 4% stacking gel was set with the appropriate number and size wells.

SDS-polyacrylamide gel electrophoresis (PAGE)

Known concentrations of protein were mixed with appropriate volumes of 5x SDS-PAGE Sample Loading Buffer and boiled for 5 min. Equal amounts of proteins were loaded in respective wells cast in the stacking gel. The resolving gels were made with 6-12% of polyacrylamide, depending on the size of the proteins to be resolved. The gels were resolved at 135 V for one hr in 1x SDS PAGE running buffer using a Mighty Small II system (Hoefer) with a protein molecular weight marker

Immunoblotting

Cell lysates in sample buffer were loaded onto gels and the proteins separated at a constant voltage of 100-250 V with a protein molecular weight marker (Rainbow markers, Amersham Life Science, UK or Prestained Protein Marker Broad Range, Biolabs). Equal amounts of protein were loaded in each lane as determined by the BioRad assay system, unless otherwise stated. After the samples were separated through the gel, the gel was transferred to a wet transfer unit and the proteins blotted onto nitrocellulose membrane (Schleicher and Schull, Germany) for one-three hrs at a constant voltage of 55 V or 20V overnight in a Hoefer Transphor Electrophoresis unit. The membrane was then stained with Ponceau S solution to determine the success of the transfer of proteins and equal loading of the lanes. The membranes were then washed in water and incubated in 10% non-fat milk at room temperature for 40-60 minutes. The membranes were then ready to be probed with primary antibody at the recommended concentrations for 1-3 hr at room temperature or overnight at 4 °C. The blots were washed with large amounts of water before addition of the secondary HRP-conjugated antibody at the recommended concentration (generally 1:2000) at room temperature for 1 hr. After incubation with the secondary antibody the membrane was washed with 1x TBS-T extensively with repeated changes of TBS-T. The ECL was then performed according the manufacture's instructions (Amersham Life Science, UK). The membrane was covered with Saran WrapTM and exposed to HyperfilmTM (Amersham Life Science, UK) for varying lengths of time to obtain an optimal exposure. If reprobing with another primary antibody was required, stripping of blots was performed. Blots were incubated with stripping buffer and freshly added mercaptoethanol in a flat bottomed tray at 55°C on a shaker for 30 minutes or with the commercial stripping buffer (Chemicon Internaional, USA) for 15 minutes at room temperature. The blots were extensively washed with TBST and then blocked in 10% milk for 1 hour at room temperature. The blot was then reprobed with primary antibody as before.

Immunoprecipitation (with cell lysates)

Cells grown in monolayers were washed three times with 1X PBS and lysed in NP40 lysis buffer or Ras IP buffer with protease inhibitors (150-250 μ l per 10 cm dish). The cells were scraped with a sterile disposable cell scraper (Greiner) and transferred to an eppendorf tube and centrifuged at 14000 rpm, 4°C for 10 minutes (eppendorf 5417R). The resulting lysate was removed to a fresh eppendorf tube and the cell debris discarded. The protein concentration was determined using the BioRad assay system. 500-2000 μ g of cell lysate was precleared with 40 μ l of 70% packed protein G beads (equilibrated in PBS buffer) for 30-60 minutes at 4°C on an eppendorf rotating wheel. The lysate was spinned at 14000rpm for 15 seconds and the supernatant was removed and replaced into a fresh tube. 20-40 μ l of antibody prebound to protein G beads was then added to the pre-cleared lysate. The mixture was left on an eppendorf rotating wheel overnight at 4°C. The protein G beads were collected by centrifugation at 3000 rpm for 4 min and the supernatant discarded. The beads were washed with three successive changes of NP40 lysis buffer or Ras IP buffer. After removing as much residual supernatant as possible, the IP beads were mixed with 20-60 μ l of 5X sample buffer and heated at 96°C for 5 minutes. The beads were centrifuged at 14000 rpm for 15 seconds and all or part of the sample loaded onto a SDS-PAGE gel. The separated proteins were Western blotted as described above.

Protein staining on acrylamide gel

Samples were resolved on an SDS-PAGE gel as described and the gel was then washed three times for 5 minutes with deionized water. Gelcode (Pearce, UK) was added to the gel (enough volume to cover it) and left to rock gently at room temperature for 15-90 minutes, until the protein bands are visible. The gel was then washed extensively with large amounts of deionized water, then dried on a gel drier (BioRad, Model 443 or 583) for 60-90 minutes at 80°C.

Small scale purification of GST-fusion proteins

BL21 or DH5 α bugs were transformed with plasmids containing the GST-tagged gene of interest. The day after the transformation 3-6 colonies were picked and grown in 4 ml 2x TYE and ampicillin. Protein expression was either induced in an aliquot of 1 ml with 0.2mM IPTG (Sigma) for 2-4 hours or not induced as a control. The cells were pelleted,

sonicated and spun at 14 000rpm (5417R centrifuge) at 4°C for 10 minutes. 20 µl of the supernatant was added to 6x SDS-PAGE sample buffer and resolved on an acrylamide gel. A western blot was performed for the protein of interest and the colony expressing the largest amount of protein after IPTG induction and having the least leakage was used for a large-scale purification.

Large scale purification of GST-fusion proteins

The colony of interest was grown in 250 ml 2xTYE with ampicillin overnight in a shaker at 37°C. The next morning the bacteria were divided 1:10 and grown for 1 hour at 37°C. 0.2 mM IPTG was used to induce protein expression for 3 hours at 30°C. The cells were pelleted for 10 minutes at 4000g, 4°C (Sorvall RC 5C Plus, rotor SLA-3000) and resuspended in 50 µl per 1 ml of culture in either cold PBS or in the case of Ras in cold GST lysis buffer. The resuspended cells were sonicated (Sonicator Ultrasonic Processor XL, Heat Systems) 3 times for 45 seconds with 1 minute between each burst and kept on ice throughout sonication. The lysate was spun at 10 000g for 10 minutes at 4°C and the supernatant was poured in a 15 ml falcon tubes. 500 µl of Glutathionine Sepharose® 4B beads (Pharmacia Biotech AB, Sweden) were washed in PBS or GST lysis buffer three times (3000 rpm, 3 minutes in a 5417R centrifuge) and resuspended in 500 µl of the appropriate buffer. The resuspended beads were added to the supernatant and inverted for 30-60 minutes at 4°C. After binding the beads were spun down, the supernatant removed and the beads washed 3 times with either 5 ml of PBS or GST lysis buffer without PMSF to remove unbound proteins.

Removing purified protein from GST tag with thrombin

Approximately 500 µl of the purified GST-protein attached to the Glutathionine Sepharose® 4B beads (Pharmacia Biotech AB, Sweden) were resuspended in an equal volume of thrombin buffer. Bovine thrombin (Sigma) was added at a concentration of 5 units/ml and incubated with inversion overnight at 4°C. The beads were spun briefly and the supernatant was transferred to a fresh microfuge tube. The beads were washed with 500 µl of thrombin buffer and the supernatant was combined with the first supernatant. 10 µl of P-aminobenzamidine-agarose beads (Sigma) was added to the supernatant to remove the thrombin and the sample was incubated with inversion for 30 minutes at 4°C. The beads were spun briefly and the supernatant transferred to a fresh

tube. The supernatant was then dialyzed overnight in 5 litres of dialysis buffer in the cold room. After dialysis, the supernatant was concentrated in centricon 10 tubes (Millipore) by spinning at 3700 rpm in Beckman Coulter for 65 minutes at 4°C. To collect the concentrated, cleaved purified protein the tubes were inverted and spun at 2000 rpm for 2 minutes. A sample of each stage was resolved on an SDS-PAGE gel and stained with Gelcode (Perbio Science, UK) to check the efficiency of cleavage and removal of thrombin.

Loading Ras with GDP/GTP

2.5 µg of recombinant protein purified from *Escherichia coli* was incubated in a total volume of 320 µl assay buffer containing 2 µCi [³H] GDP or [³H] GTP in a water bath for 10 minutes at 30°C. 2 µl of each sample was put on a blot paper and was added to 5 ml scintillation liquid (Ecoscint A, National Diagnostics), inverted several times and left to stand for 2 hours. Its tritium content was measured using the LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter, USA) to check the equal loading of GDP and GTP.

2.2.5. Cell-based assays

Cell transfection

Adherent cells were split to a confluence of between 70-80% in fresh medium. Typically a 60 mm dish had 7×10^5 cells of Saos2 or 4.5×10^5 of U2OS cells plated 24 hours prior to the transfection procedure. 15-30 minutes before the transfection the medium on the dishes was replaced with 2.5 ml of fresh medium. 2X HBS buffer was diluted in sterile water to a concentration of 1X and a volume of 250 µl per 60 mm dish placed in sterile eppendorf tubes. The required amount DNA was added using sterile Gilson tips and mixed thoroughly. To form the precipitate, 12.5 µl of 2.5 M CaCl₂ was added to the transfection mix and left at room temperature for 12-15 minutes. After this time the mixture was added drop-wise in the tissue culture hood to the cells and the dishes replaced in the incubator for 6 hours. The medium was removed and a wash of DMEM medium without fetal calf serum was applied to the cells. Finally 5 ml growth

tissue culture medium was added to each dish and the cells were left for 24 hours as a transient assay.

Flow cytometry analysis

The method was carried as described before (Hsieh et al., 1997a). Briefly, transfection was carried out using the calcium phosphate method. 2×10^6 Saos-2 cells were seeded 48 hours before transfection. They were transfected with the respective amounts of plasmid, normalised with empty vector and co-transfected with the selectable CD20 marker with one control dish not transfected with CD20. After 36 hrs both attached and floating cells were harvested using 4 mM EDTA, washed and pelleted by centrifugation at 490 g. Cells were then resuspended in complete medium containing FITC-conjugated anti-CD20 antibody (Becton Dickson, CA, USA) and incubated for one hr on ice in the dark. The cells were then washed once with PBS and fixed with 70% methanol overnight at 4°C. The methanol was washed away and the cells resuspended in 1x PBS containing propidium iodide (50 µg/ml) and RNase (100 µg/ml) (Sigma) in the dark for one hr at room temperature. The DNA content of all the cells were analysed by FACS (FACSort, Becton Dickson) as described [Hsieh *et al.*, 1997].

Immunocytochemistry

Monolayers of cells were grown in on sterile glass coverslips in 24-well plates or 30 mm dishes. The cells were washed with 1x PBS and fixed with an appropriate volume of 4% para-formaldehyde for 15 minutes at room temperature and then washed in 1X PBS. 0.2% Triton-X100 in 1X PBS was used to permeabilise the cells for 2 minutes at room temperature and this was washed off with three washes of 1X PBS. Primary antibody was prepared in tissue culture medium containing azide at the appropriate concentration and added to the dishes for 1-3 hours at room temperature. The dishes were washed with 1X PBS and the secondary antibody of either anti-rabbit or anti-mouse FITC (Fluorescein isothiocyanate) or TRITC (Tetramethyl rhodamine isothiocyanate) prepared in tissue culture medium at the manufacturers recommended dilution (Jackson labs) and added to the dishes for 30-60 minutes. The cells were washed in 1X PBS and left to air dry. Citifluor shielding agent (Citifluor, UK) or mowiol was applied as a drop to the slide and the cover slips with the cells attached are placed on top (cells facing the citifluor/mowiol). A drop of immersion oil on top of the

cover slip allowed the immunocomplexes to be visualised using a fluorescence microscope (Nikon). Images were captured on film and thus presented. Alternatively, immunocomplexes were visualised using a converted fluorescence microscope.

***In vivo* Transcription assays**

7×10^5 Saos-2 cells or 4.5×10^5 U2OS cells were seeded in 60mm dishes and transfected 24 hrs later using the calcium phosphate precipitation method with the various expression plasmids, including a luciferase reporter plasmid. Twenty-four hours after transfection, cells were washed twice with PBS, lysed in 150 μ l 1x Reporter Lysis Buffer, scraped with a sterile disposable scraper (Greiner) and put in an eppendorf tube. The lysate was left on ice for 15 minutes before spinning them at 14000 rpm for 30 seconds. 20 μ l of supernatant was then placed in a wash tube (Sarstedt, Germany) and its luciferase activity measured in an automated Luminometer (AutoLumat LB 953, EG&G, Berthold) using the Luciferase Assay Kit (Promega, USA). The mean values were calculated from at least two independent experiments.

Protein stability analysis by cyclohexamide treatment

Cells were either transfected with the appropriate expression plasmids or treated with EGF and UO126 as indicated, the day prior to cyclohexamide treatment. Cyclohexamide was added to cells at a final concentration of 50 μ g/ml in the appropriate medium. Cells were harvested and lysed with the Ras IP buffer (with protease and phosphatase inhibitors) at the time points indicated. A Western Blot was performed with an aliquot of the lysates as described above.

Pulse-chase

Saos2 were seeded in 10 cm dishes and transfected by calcium phosphate with V5-tagged ASPP2 wild type or mutant in pCDNA expression plasmids and K-RasV12 in pEF expression plasmids as indicated. 6 hours after transfection, the precipitated were washed and DMEM with 0.5% FCS was added overnight. The next morning a DMEM solution without cysteine, methionine or glutamine (Sigma) was complemented with non-radioactive glutamine and 35 S-labelled Methionine and Cysteine (ICN Biomedicals) and at a final concentration of 145 μ Ci/ml. This solution was added to the Saos2 cells for 2 hours in a 37°C incubator with 10% carbon dioxide. The radioactive medium was

removed from the cells and the cells were washed 3x with PBS before adding normal DMEM with 0.5% FCS. Cells were harvested and lysed in luciferase buffer (Promega) at the times indicated. The lysates were pre-cleared with sepharose-G-beads for 30 minutes at 4°C. Pre-bound V5-sepharose-G-beads were then added to the lysates for 2 hours at 4°C to immunoprecipitate transfected ASPP2. The V5-G-beads were then retrieved and PC-10-sepharose-G-beads were added to the lysates for 1 hour at 4°C to immunoprecipitate endogenous PCNA. The beads were washed with three successive changes of NP40 lysis buffer. After removing as much residual supernatant as possible, the IP beads were mixed with 25 µl of 5X sample buffer and heated at 96°C for 5 minutes. The beads were centrifuged at 14000 rpm for 15 seconds and all or part of the sample loaded onto a SDS-PAGE gel. The separated proteins were Western blotted as described above.

Ultraviolet light treatment (UV)

Cells were grown to sub-confluency and the medium removed and retained in a tissue culture hood. The monolayer of cells was exposed to 10 J M² of ultraviolet light using a spectrolinker XL-1500 UV crosslinker (Spectronics Corporation). The medium was replaced on the cells and the dishes were placed back in a 37°C incubator with 10% carbon dioxide. The cells were harvested after the appropriate time points.

2.2.7. *In vitro* assays

***In vitro* translation of plasmids**

In vitro transcription and translation of plasmids were performed using the Promega TNT® T7 Quick coupled Transcription /Translation system. A typical reaction was carried out using 40 µl of Reaction mix containing rabbit reticulocyte lysate, reaction buffer, all amino-acids (except methionine), RNase inhibitors and T7 RNA polymerase together with 1 µg of plasmid containing the T7 promoter. Either 2 µl of ³⁵S-Methionine (for radio-labelled proteins) or 1 mM Methionine (for non-labelled proteins) was added to the reaction mix and made up to 50 µl with nuclease-free water. This was incubated at 30 °C for 60-90 min.

Preparation of Protein G beads

Protein G Sepharose™ 4 Fast Flow beads (Amersham Pharmacia Biotech AB, Uppsala, Sweden) were washed three times with excess volume of PBS then resuspended in PBS at a ratio of 1:1. For immunoprecipitations, the G-beads were pre-bound to the required antibody by mixing 300ml of the 50% slurry protein G -beads, 500ml of DMEM with 10% serum and 30 ml ascites or the required amount of commercially-available solution in a 1.5 ml tube. This was incubated on a rotating wheel for at least 2hrs before the immunoprecipitation and then washed three times with PBS.

***In vitro* binding assays**

The plasmids were *in vitro* translated as described above or the recombinant protein purified as described above. If the binding was between a cold *in vitro* translated protein and a radioactive *in vitro* translated protein, then 2µl of RNase (100 µg/ml, Sigma) and 2 µl of DNase (10 units/µl of DNase I, Roche Diagnostics, UK) was added to 50 µl of the radioactive sample for 30 minutes at 30 °C before the two samples were mixed. 5-40 µl of reactions lysates of each *in vitro* translated product were mixed and allowed to bind at 30 °C for 1 hour. For recombinant protein, 1.5 µg of protein was used. Lysates were pre-cleared using 30 µl of 50% G-bead slurry for 1 hr on a rotating wheel at 4 °C. The supernatant of the binding reaction was transferred to a fresh Eppendorf tube and 30 µl of the pre-bound G-beads was added and left overnight on the rotating wheel at 4 °C. The beads were pelleted by a 4min centrifugation at 3000 rpm (Microcentur, MSE) at 4 °C, and washed three times with 500 µl PBS buffer. The beads were resuspended in 20-40 µl 5x SDS-PAGE sample buffer and boiled for 5 min to release the bound proteins, centrifuged briefly at 14 000 rpm (Eppendorf 5415C) and then loaded on an SDS-PAGE gel for analysis.

Small scale *in vitro* phosphorylation assay

Purified recombinant GST-ASPP2 (693-1128) was produced as described above at a concentration of 1mg/ml in 1x kinase buffer. 3 µl of the 10x Tris solution (50mM Tris-HCl pH 7.5, 0.1% mercaptoethanol) was mixed with 3 µl phosphatase inhibitor microcystine (final concentration 1 µM). Approximately 0.03 units of kinases p38 SAPK, PKA, PB, MAPK1, p70 and p90 were added. In the case of PKB, 2 µl of

reduced glutathione (0.2 M) was also added to the tube. This solution was mixed with either 2 µg of GST-ASPP2 (693-1128), 2 µg of 1mg/ml recombinant H2B or 2 µl of water. Water was added so that the total volume was 24 µl. Separately, 6 µl of a 1:1 ratio Magnesium Acetate-ATP (either cold or ³²P-labelled at 500 cpm/pmol) was prepared and subsequently added to the kinase mixture and put in a waterbath at 30°C for 30 minutes. 10 µl of sample buffer and DTT (final concentration 25 mM) was added to stop the reaction. The samples were boiled for 2 minutes and resolved on an SDS-polyacrylamide gel.

Large scale *in vitro* phosphorylation assay

This assay was performed in an identical manner to the small scale *in vitro* phosphorylation assay except for the following: The ³²P-labelled ATP had 10000cpm/pmol and the final concentration used was 100 µM. Fewer kinases were used and they were used at a higher concentration, namely 0.15 units of PKA, 0.35 units MAPK1, 0.1 units p38 SAPK and 0.3 units p90 rsk. After the 30 minute incubation in the 30°C waterbath the reaction was stopped with sodium dodecyl sulfate (final concentration 1%) and DTT (final concentration 25 mM). After boiling for 2 minutes, 4-vinyl pyridine was added to a final concentration of 50 mM and the samples were put back in the 30°C waterbath for another 30 minutes. Sample buffer without any DTT was then added and the samples were resolved on an SDS-PAGE gel. The gel was stained with coomassie. To destain the gel, destaining buffer was added and heated for 20 seconds, the gel was left for 2-3 minutes to incubate in the warm buffer and discarded; this was repeated approximately 6 times for full destaining of the gel. The gels were exposed with a film and the film directed where the bands were to cut them out of the gel. The samples were brought to the keratin-free area. 1ml of MilliQ was added to the gel fragments and left to shake for 15 minutes at room temperature. The supernatant was removed and 1 ml of 50% acetonitrile in water was added to the gel and left to shake for 15 minutes at room temperature. The supernatant was removed before adding 1 ml of 0.1 M ammonium bicarbonate to the gel for 15 minutes at room temperature, shaking. The supernatant was once again removed and 1 ml of a solution containing 0.05 M ammonium bicarbonate / 50% acetonitrile was added to the gel for 15 minutes at room temperature. The supernatant was removed and the gel mashed with a mini teflong stick (1 stick per sample). 1 ml of 100% acetonitrile was added for 5 minutes at

room temperature. As much of the supernatant as possible was removed before putting the sample in a speed vacuum for 5 minutes to dry. 1ml of the trypsin solution was added to 16 μ l of trypsin. 200 μ l of this was added to each desiccated gel and left at 30°C at room temperature, stationary. Another 200 μ l of the trypsin solution without the trypsin was added and the samples were left on a shaker overnight at 30°C.

Detection of immuno-complexes

For detection of non-labelled proteins, immunoblotting was used as detailed before. For detection of radio-labelled proteins, the gel was fixed in Fixer Solution for 15-30 minutes at room temperature and then incubated for 30 min with Amplify™ (Amersham Pharmacia Biotech) solution also at room temperature. This was then dried on a gel drier (BioRad, Model 443 or 583) at 80 °C for 90-120 min before exposure to an X-ray sensitive film overnight or exposure to a phosphoscreen (Molecular Dynamics) which was then scanned on a phosphoimager (Molecular Dynamics) using STORM860 software.

2.2.8. Data Analysis

Quantification of intensity of western blot signal

For quantification of the signal, the GenomeTM machine was used to digitally capture the image and quantification carried out using SynGene software (SynGene, UK). Relative values were assigned to each peak, corresponding to the intensity of the light emitted by the ECL method, by giving the background (vector only lane) a value of one.

Computer images

All autoradiographs were scanned using the UMAX power Look II scanner and the Adobe photoshop 5.5 software. Images were manipulated only as a whole size, brightness and contrast. No signal was modified in relation to the whole image.

Data presentation

The mean and standard deviation between experiments are presented where applicable.

Chapter 3

Activated Ras binds to and stimulates the activity of ASPP1 and ASPP2

3.1 Introduction

p53 is the most commonly mutated tumour suppressor protein thus far identified. Its role in preventing tumour formation has been highlighted by studies in a *p53* knock-out mouse system where the mice were shown to develop spontaneous tumours at an early age (Donehower et al., 1992). The importance of p53 in protecting cells from uncontrolled proliferation also applies in humans: Li-Fraumeni disease is caused by a mutation in one of the alleles of *p53* and affected individuals have a strong predisposition to develop cancers (Srivastava et al., 1990).

In normal cells, p53 protein levels are low due to its short half-life. Stress signals, such as telomere erosion, hypoxia, loss of survival signals and DNA damage can result in increased p53 levels, which is mediated by post-translational modifications such as phosphorylation, acetylation and sumoylation (Brooks and Gu, 2003; Meek, 1999; Melchior and Hengst, 2002; Xu, 2003). Oncogenes, such as Ras, myc and E1A are also known to activate p53 via their ability to induce the expression of *Arf* and prevent Mdm2 mediated protein degradation of p53 (de Stanchina et al., 1998; Palmero et al., 1998; Zindy et al., 1998).

Activated p53 is promiscuous in its responses to stress signals: it can induce differentiation, DNA repair and senescence. However the two most common responses are cell cycle arrest and apoptosis. Most of the p53-dependent responses are mediated by its ability to transactivate downstream effector genes by recognition of sequence-specific binding sites in their promoter region (Prives and Hall, 1999), although p53 can

also repress transcription of a number of genes (Ginsberg et al., 1991; Ho and Benchimol, 2003; Kley et al., 1992; Subler et al., 1992). p53 mediates cell cycle arrest by the transactivation of *p21*, *14-3-3 σ* and *GADD45* (El-Deiry et al., 1993; Hermeking et al., 1997; Kastan et al., 1992b) and others. The mechanisms of inducing apoptosis are less clear and several downstream effectors are involved, including *BAX*, *PIG3*, *PUMA*, *FAS*, *NOXA*, and *PERP* to name but a few. p53 has also been shown to induce apoptosis in a transcription-independent manner (Caelles et al., 1994; Haupt and Oren, 1996).

Although p53 is the tumour suppressor protein most frequently found to be mutated in cancers, its mutation rate varies between different types of human cancer ranging from 5% in leukaemia to 70% in lung cancer to 100% in medullary breast cancer. In tumours carrying *Ras* mutations, *p53* is generally mutated too, for example in pancreatic, colon and lung tumours. In colorectal tumour models, *Ras* mutation is an early event while *p53* mutation occurs predominantly in metastatic tumours. This suggests that there might be a selective advantage in tumours expressing mutant *Ras* to inactivate the tumour suppression function of p53.

Recent studies demonstrated that oncogenic *Ras* can induce senescence and this is mediated by p53 and its downstream target gene *p21* (Pantoja and Serrano, 1999; Serrano et al., 1997; Weber et al., 1999). However the tumour suppression function of p53 is most closely linked to its ability to induce apoptosis. It remains unclear whether oncogenic *Ras* plays a role in regulating p53-mediated apoptosis.

The mechanism by which p53 differentiates between different cellular responses remained elusive until the discovery of p53 co-factors that could stimulate specific p53-

responses. ASPP1 and ASPP2 are two of these co-factors that have been shown to increase p53 transactivation of its pro-apoptotic effectors specifically (Samuels-Lev et al., 2001).

The ability of ASPP1 and ASPP2 to stimulate p53-dependent apoptosis requires full length proteins since mutants lacking the first 120 amino acids are unable to co-activate p53. A BLAST search was performed with the amino terminal region of the ASPP proteins and they were found to contain a putative Ras-association domain. In this chapter, I show that Ras can bind ASPP1 and ASPP2 and that, in so doing, it stimulates the ability ASPP to induce p53-dependent apoptosis.

3.2 Results

3.2.1. The amino terminus region of ASPP2 is necessary for its full activity

ASPP1 and ASPP2 are known to have several regions of amino acid homology. The carboxy terminus contains a proline-rich region, an ankyrin repeats and an SH3 domain. The latter two have been shown to interact with p53 *in vitro* and *in vivo* (Iwabuchi et al., 1994; Samuels-Lev et al., 2001). The other region of homology between the two proteins is in their amino terminal domain. This region contains a predicted α -helix. Interestingly, this amino-terminal region of ASPP2 is necessary for its full activity as a mutant ASPP2 lacking the first 123 amino acids was not able to increase p53 transactivation activity to the same extent as wild-type ASPP2 (figure 3.1). This confirms work that had previously done in our laboratory that also showed that an ASPP1 mutant lacking the amino terminal region is also not as active as the wild type ASPP1 in stimulating p53 transactivation (Samuels-Lev et al., 2001).

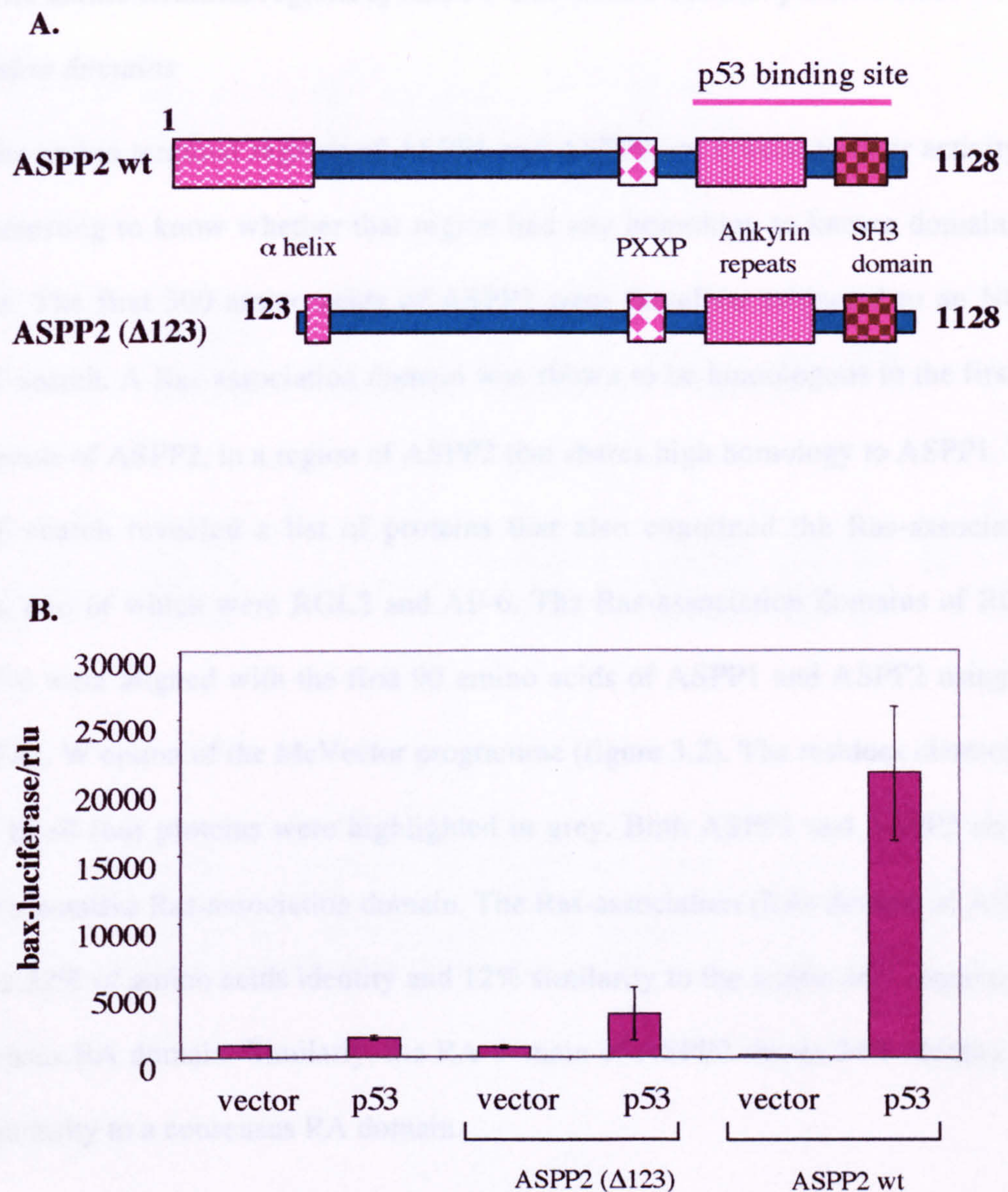


Figure 3.1 The amino-terminal 130 amino acids of ASPP2 is crucial for its full activity. (A) Schematic representation of ASPP wt (top fragment) and the mutant ASPP2 lacking the first 123 amino acids (ASPP2 Δ123), bottom fragment. Both fragments contain a proline rich region (PXXP), ankyrin repeats and an SH3 domain in their carboxy-terminus. (B). Saos2 cells were transfected in 6 cm dishes with 1 μg bax-luciferase, 50 ng p53 and 4 μg of ASPP2 wt or ASPP2 Δ123 as indicated. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out.

3.2.2. The amino terminal regions of ASPP1 and ASPP2 contain putative Ras-association domains

Since the amino terminal regions of ASPP1 and ASPP2 are crucial to their activity, it was interesting to know whether that region had any homology to known domains or proteins. The first 300 amino acids of ASPP2 were therefore subjected to an NCBI BLAST search. A Ras-association domain was shown to be homologous to the first 90 amino acids of ASPP2, in a region of ASPP2 that shares high homology to ASPP1. The BLAST search revealed a list of proteins that also contained the Ras-association domain, two of which were RGL2 and AF-6. The Ras-association domains of RGL2 and AF-6 were aligned with the first 90 amino acids of ASPP1 and ASPP2 using the CLUSTAL W option of the McVector programme (figure 3.2). The residues identical or similar in all four proteins were highlighted in grey. Both ASPP1 and ASPP2 clearly contain a putative Ras-association domain. The Ras-association (RA) domain of ASPP1 contains 22% of amino acids identity and 12% similarity to the amino acid sequence of a consensus RA domain. Similarly, the RA domain of ASPP2 shares 24% identity and 11% similarity to a consensus RA domain.

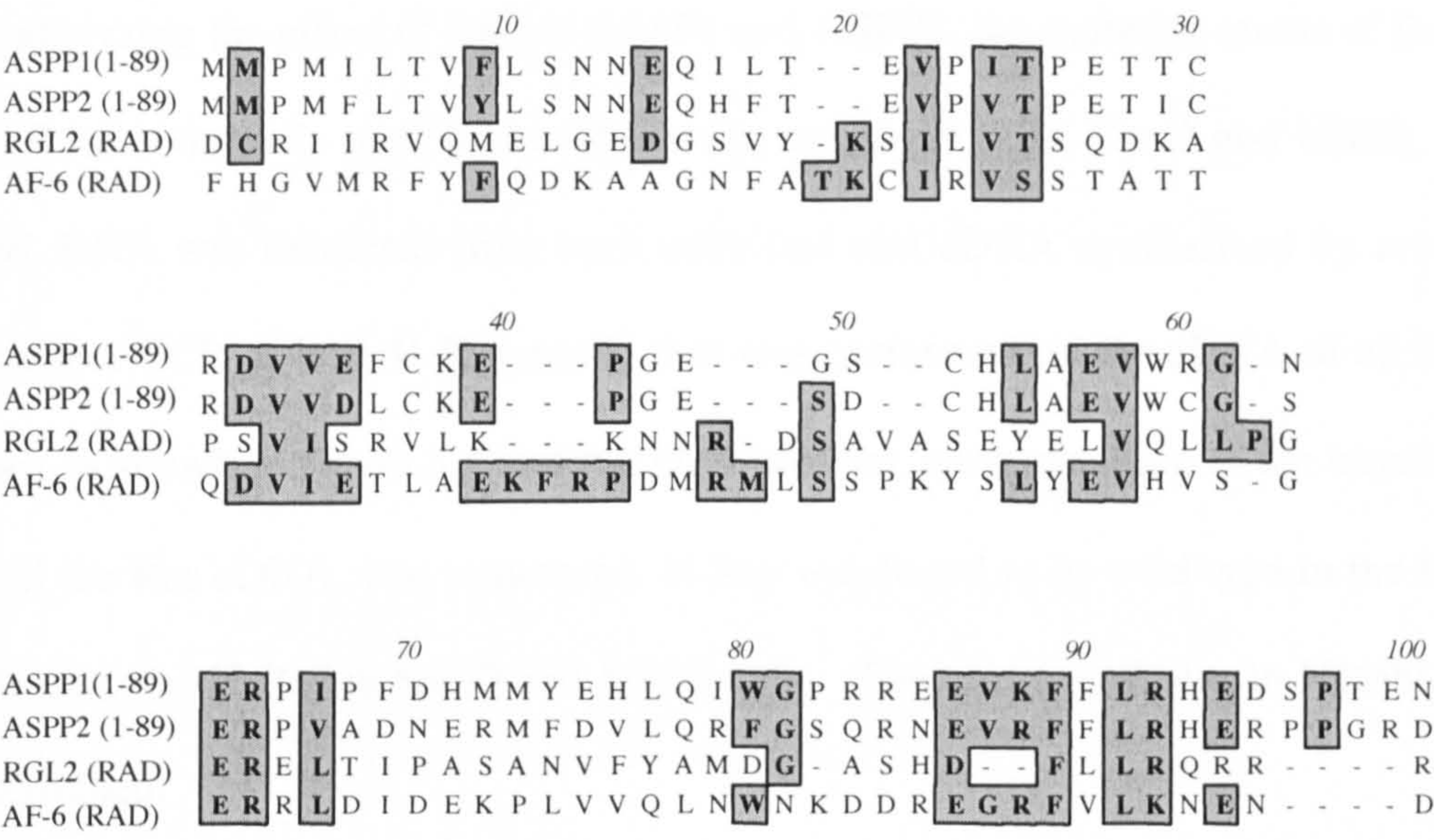


Figure 3.2 *ASPP1* and *ASPP2* contain a putative *ras*-association domain in their amino-termini. The first 89 amino acids of *ASPP1* and *ASPP2* are aligned with the *ras* association domain (RAD) of the human *RGL2* and *AF-6* proteins. Residues that are identical or similar in those four proteins are highlighted in grey. The figure reveals that *ASPP1* and *ASPP2* have homology to the RA domain of *RGL2* and *AF-6*.

3.2.3. Ras mutation status in H1299, U2OS and Saos2 cells

Before analyzing the effect of Ras on ASPP1 and ASPP2, the mutation status of Ras in the cell lines commonly used in the laboratory, namely H1299, Saos2 and U2OS, was checked. RNA was extracted from each cells line and cDNA synthesised by reverse transcription. PCR of both H-Ras and K-Ras was performed on the cDNA of all three cell lines as shown in figure 3.3 and the PCR product, corresponding to the translated region of the Ras cDNA, was sequenced. H-Ras was found to be wild type in the three cell lines, as was K-Ras in Saos2 and U2OS cells. The status of K-Ras in H1299 was not determined.

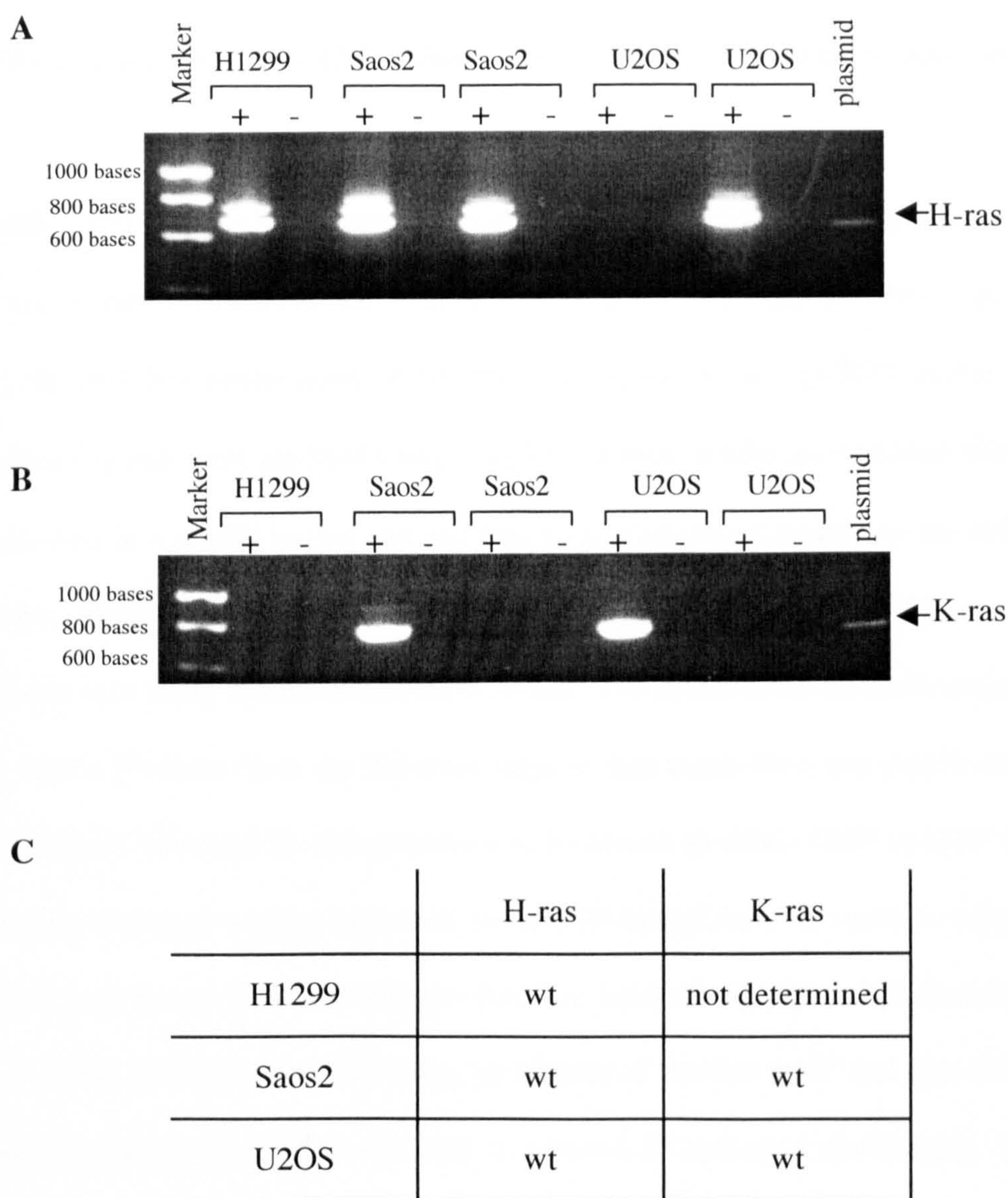


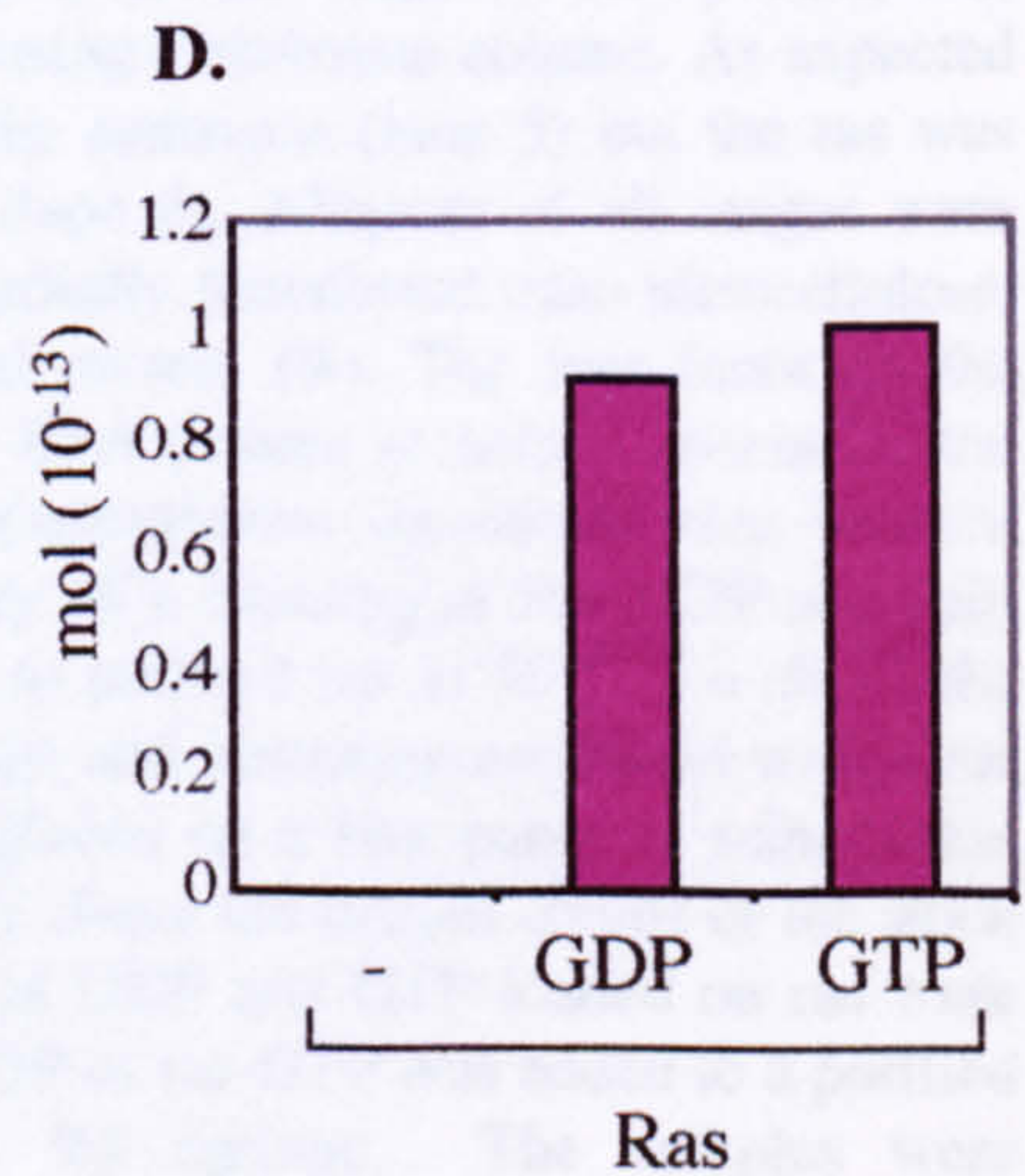
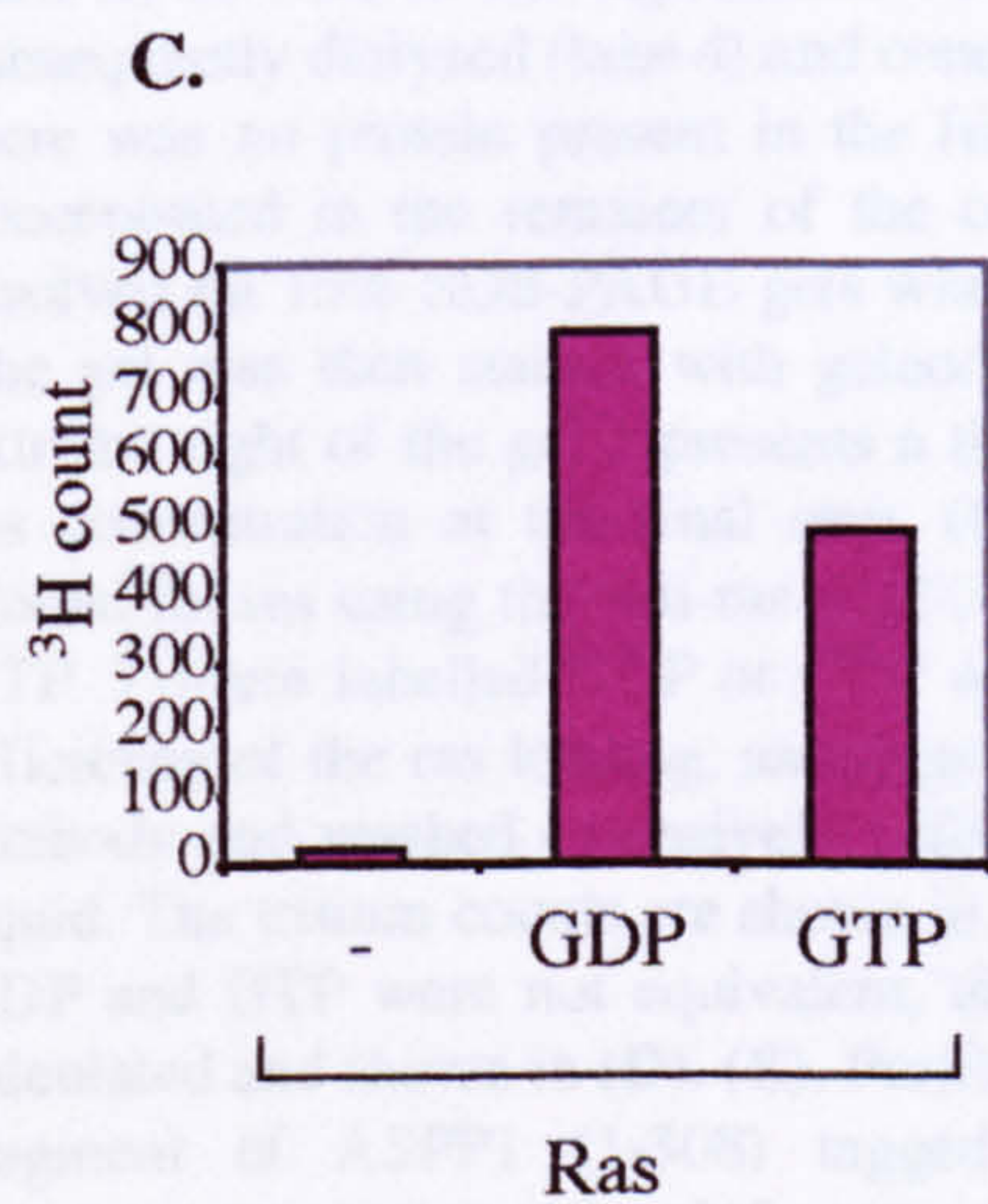
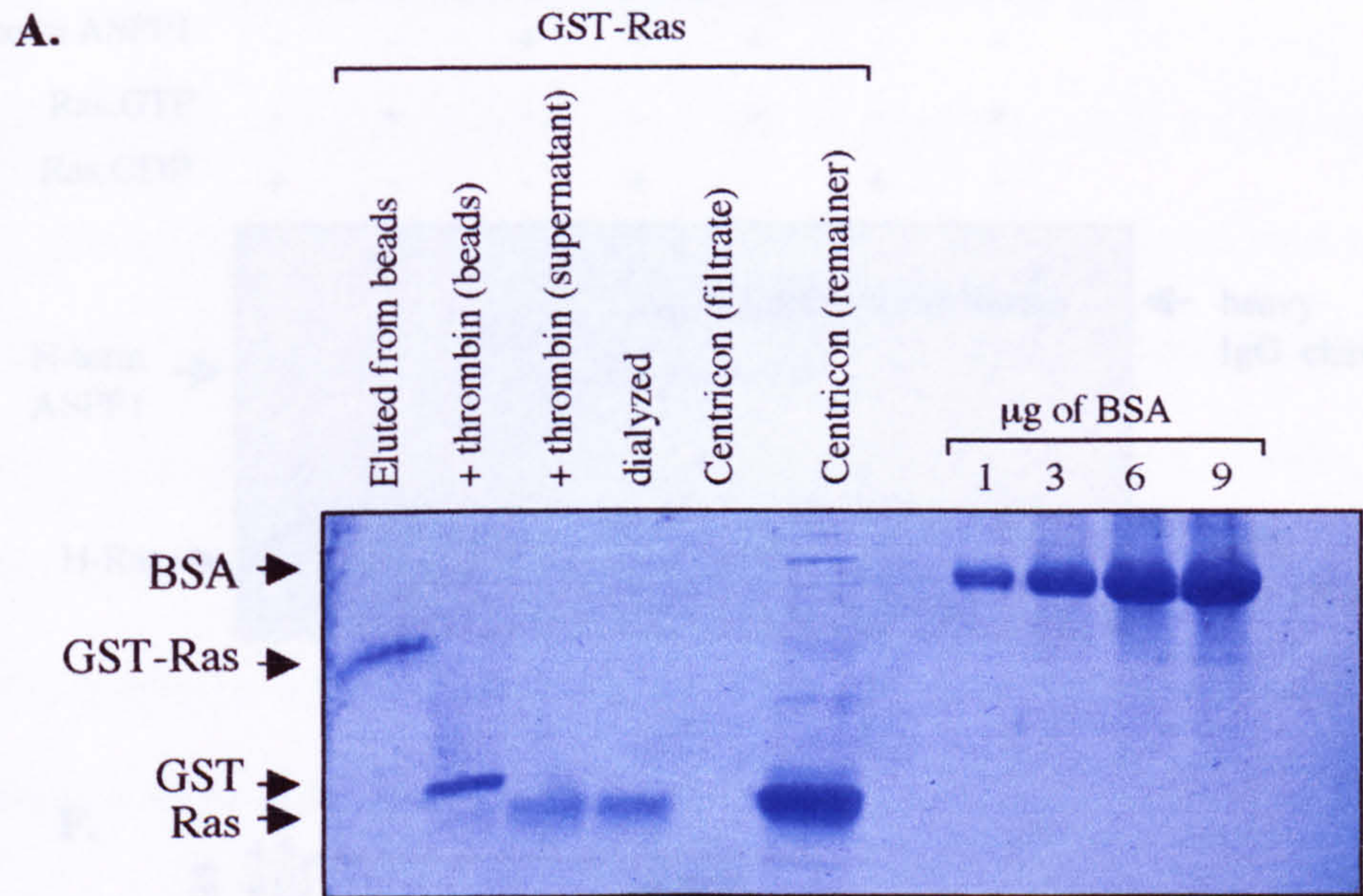
Figure 3.3 *Ras* mutation status in H1299, Saos2 and U2OS. (A) and (B) Total RNA was isolated from H1299, Saos2 and UO2S and cDNA was synthesised by reverse transcription. As a negative control, the identical procedure was performed without any reverse transcriptase. PCR of H-ras (A) and K-ras (B) was performed on the RNA with reverse transcriptase (“+”) and without reverse transcriptase (“-”) and the PCR products resolved on 2% agarose gels. The left lane is the 100bp DNA marker and the right lane represents a PCR done in the same conditions but with a control plasmid instead of cDNA. The PCR products from A and B were purified and sequenced. (C). The sequencing results of the PCR products showing the wild type vs mutant status of the H-ras and K-ras cDNA. PCR product amplification of K-ras from H1299 RNA was unsuccessful so its status in that cell line was not determined. All other PCR products shown in this figure were shown to be wild type.

3.2.4. The amino terminus of ASPP1 binds directly to Ras-GTP preferentially to Ras-GDP

Since both ASPP1 and ASPP2 have a putative Ras-association domains, the next step was to see if they could associate with Ras. In order to see whether there was direct binding, the first 308 amino acids of ASPP1 were expressed in a pCRT7 vector with a 6x histidine tag and were purified using a nickel column, whilst recombinant GST-Ras was expressed in a pGEX vector and purified with glutathione beads. As the GST can bind some proteins non-specifically, the GST epitope was cleaved off from the recombinant Ras using thrombin and the thrombin was removed by aminobenzamidine-agarose beads. Proteins from the different steps in Ras production and purification are shown in figure 3.4A and B. Ras is known to be bound to either GDP or GTP *in vivo* and generally interacts with its substrates in its GTP-bound form. In order to imitate the *in vivo* binding, the purified recombinant Ras was loaded with tritium-labelled GDP or GTP. To check the efficiency of loading, an aliquot of the Ras-GDP and Ras-GTP was immunoprecipitated with a Ras antibody to remove all unbound nucleotides. The ³H guanine nucleotide content of the immunoprecipitates was measured as shown in figure 3.4 C. To correct for the slightly different specific activities of the GDP and GTP nucleotides, the mole of GDP/GTP nucleotides loaded onto Ras was calculated and shown in figure 3.4D. The loading of GDP and GTP on to Ras was comparable. This was necessary if the binding of the ASPP1 fragment to Ras-GDP and Ras-GTP were to be compared.

The amino terminal fragment of ASPP1 (1-308) was mixed with Ras-GDP and Ras-GTP, and the V5 antibody against the tagged ASPP1 was used to immunoprecipitate the proteins. More Ras-GTP was immunoprecipitated by ASPP1 (1-308) than Ras-GDP

showing a specificity in binding (figure 3.4 E). Neither Ras-GDP nor Ras-GTP were immunoprecipitated in the control samples with no ASPP1 (1-308) present. The bands representing Ras-GDP and Ras-GTP immunoprecipitated by ASPP1 (1-308) were quantified as shown in figure 3.4 F. From this experiment, it can be concluded that the amino terminus region of ASPP1 binds directly to Ras and has a higher specificity for Ras-GTP than for Ras-GDP.



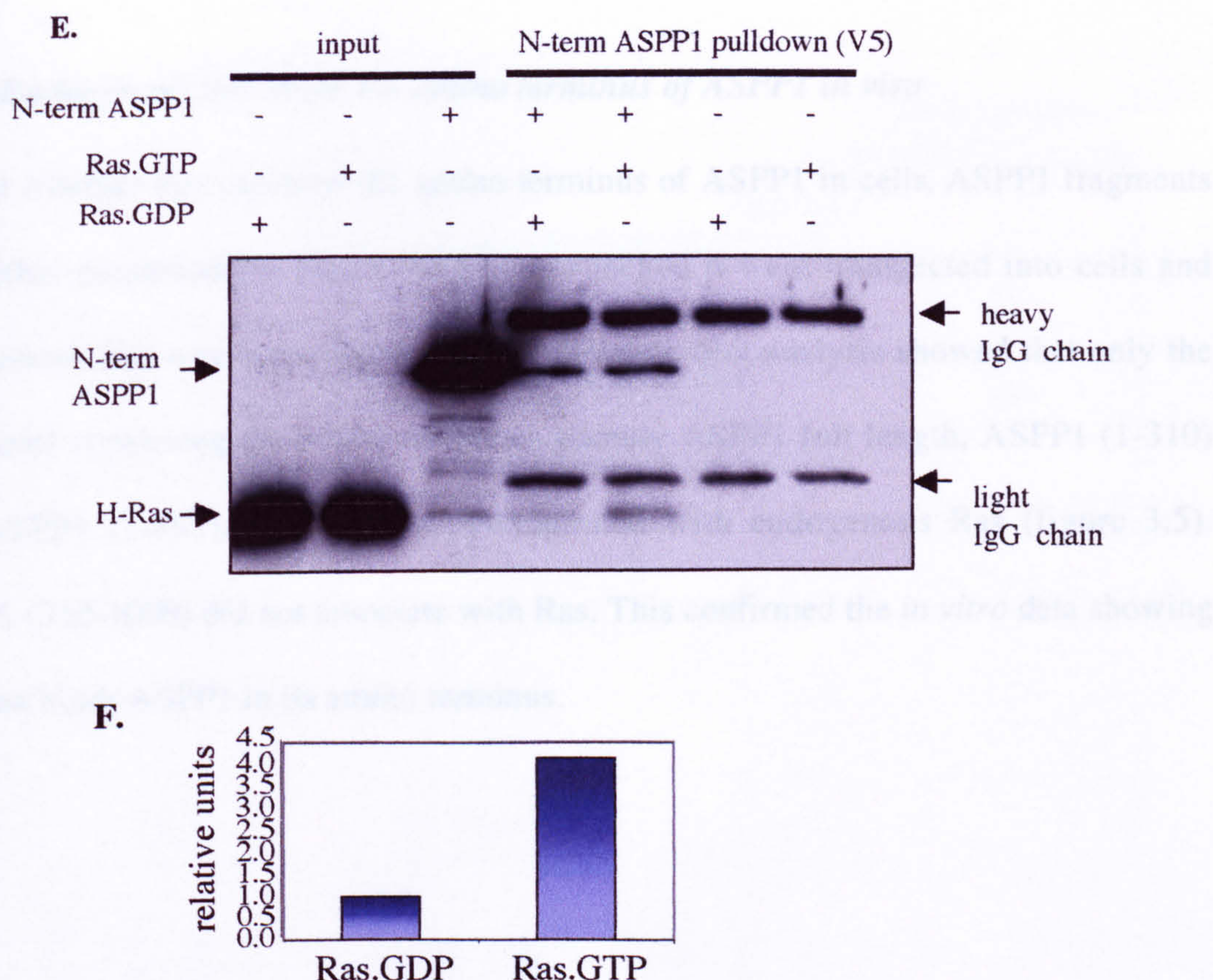
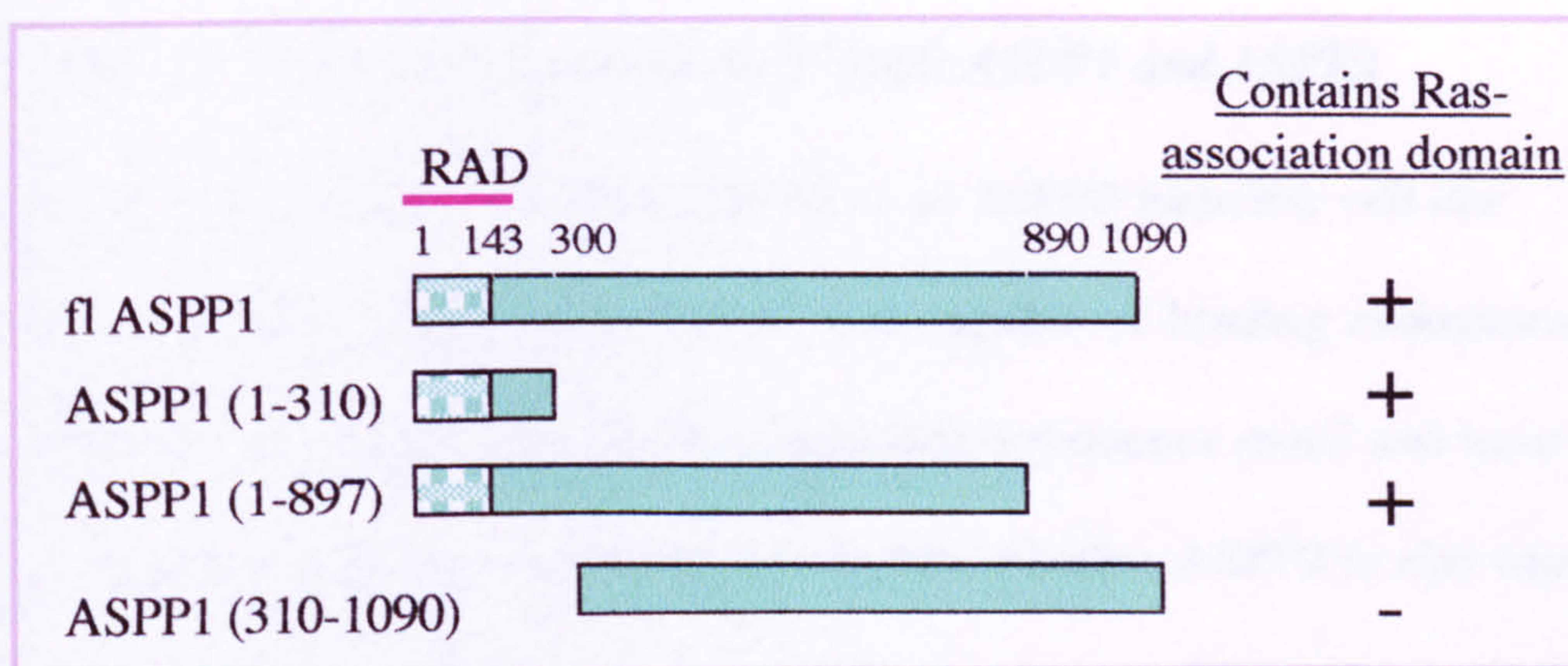


Figure 3.4 Purified amino terminus of ASPP1 binds directly to Ras-GTP with a higher affinity than to Ras-GDP. (A). A plasmid encoding GST-ras was transformed in BL21 cells. Protein expression was induced with IPTG and the protein purified with glutathione beads. GST-ras was eluted from the beads (lane 1). Thrombin was added to GST-ras to cleave the GST tag and aminobenzamide-agarose beads was present to remove the thrombin. An aliquot of the beads containing thrombin and GST is shown (lane 2) as well as the supernatant containing purified ras (lane 3). Ras protein was subsequently dialyzed (lane 4) and concentrated using a centricon column. As expected there was no protein present in the filtrate of the centricon (lane 5) but the ras was concentrated in the remainder of the centricon (lane 6). Aliquots of all stages were resolved on 15% SDS-PAGE gels which was partially transferred onto nitrocellulose. The gel was then stained with gelcode for total protein (A). The four lanes on the extreme right of the gel represents a titration of BSA protein to help approximate the ras concentration at the final step. (B). The nitrocellulose membrane was western blotted for ras using the anti-ras RO2120 antibody. (C). Loading of Ras-GDP and Ras-GTP. Tritium labelled GDP or GTP was added to purified ras at 30°C. To check the efficiency of the ras loading, an aliquot was taken and immunoprecipitated with a ras antibody and washed extensively before being placed on a blot paper in scintillation liquid. The tritium counts are shown in graph (C). Since the tritium counts of the stock GDP and GTP were not equivalent, the moles of GDP and GTP loaded on ras were calculated and shown in (D). (E). Purified ras-GDP or ras-GTP was added to a purified fragment of ASPP1 (1-308) tagged with a V5 epitope. The samples were immunoprecipitated with a V5 antibody and the samples analyzed on an SDS-PAGE gel. A western blot was performed with a Ras antibody and a V5 antibody to check the amount of ASPP1 fragment. As a control, ras-GDP and ras-GTP were immunoprecipitated with V5 in the absence of the ASPP1 (1-308) fragment as shown on the last two lanes. (F). Ras-GTP was immunoprecipitated by ASPP1 (1-308) with a higher affinity than ras-GDP. The signal of the band was quantified and the values shown as a bar graph.

3.2.5. Endogenous Ras binds the amino terminus of ASPP1 in vivo

To test whether Ras can bind the amino terminus of ASPP1 in cells, ASPP1 fragments that either contained the amino-terminus or lacked it were transfected into cells and endogenous Ras was immunoprecipitated. Western blot analysis showed that only the fragments containing the amino terminus, namely ASPP1 full length, ASPP1 (1-310) and ASPP1 (1-897), were immunoprecipitated with endogenous Ras (figure 3.5). ASPP1 (310-1090) did not associate with Ras. This confirmed the *in vitro* data showing that Ras binds ASPP1 in its amino terminus.

A.



B.

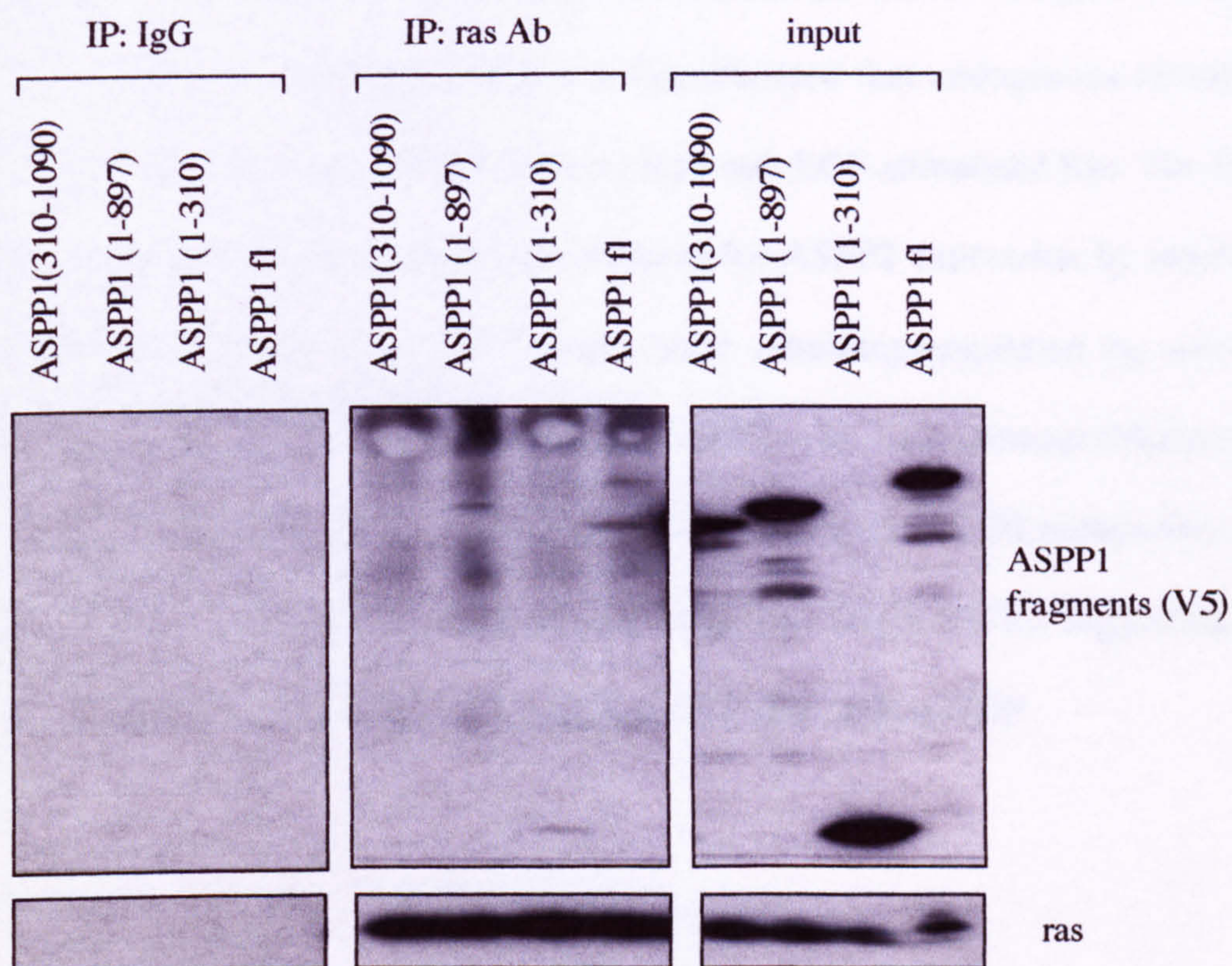


Figure 3.5 *Endogenous Ras binds the amino-terminus of ASPP1 in vivo.* (A). ASPP1 fragments were constructed, each lacking different regions of the full length protein as shown in schematic representation. All ASPP1 fragments are V5-tagged in their carboxy terminus. (B). The ASPP1 fragments shown in A were transfected in Saos2 cells and the lysates were immunoprecipitated with either ras antibody or a control IgG antibody. The immunoprecipitations were resolved on an SDS-PAGE gel. Western blotting was performed to detect ASPP1 fragments with the V5 antibodies and Ras with RO2120 antibody.

3.2.6. Endogenous Ras stimulated with EGF binds ASPP1 and ASPP2

3.2.6.1 Ras stimulated with EGF binds ASPP2 in an ASPP2-inducible cell line

As shown in figure 3.5, full length ASPP1 was capable of binding endogenous Ras. Both ASPP1 and ASPP2 contain the Ras-association sequence motif and have a high degree of sequence similarity in that region. To test whether ASPP2 is also capable of binding Ras *in vivo*, an ASPP2-inducible cell line was used. The cells were placed in medium with low serum to remove the background of Ras stimulation, and endogenous Ras was then “activated” with epidermal growth factor (EGF) and serum to switch it from its GDP to its GTP form. Since Ras-GTP bound the amino terminus of ASPP1 with a higher affinity than Ras-GDP, it was hypothesised that endogenous stimulated Ras would bind ASPP2 with a higher affinity than non-EGF-stimulated Ras. The EGF-stimulated and non-stimulated cells were induced for ASPP2 expression by removing tetracycline from the medium. The lysates were immunoprecipitated by anti-Ras antibody and western blot analysis showed ASPP2 was co-immunoprecipitated in ASPP2-induced lysates (figure 3.6). There was an increase in ASPP2 association with Ras after cells were stimulated with EGF and foetal calf serum (FCS), suggesting that ASPP2 also has a higher binding affinity to Ras-GTP than to Ras-GDP.

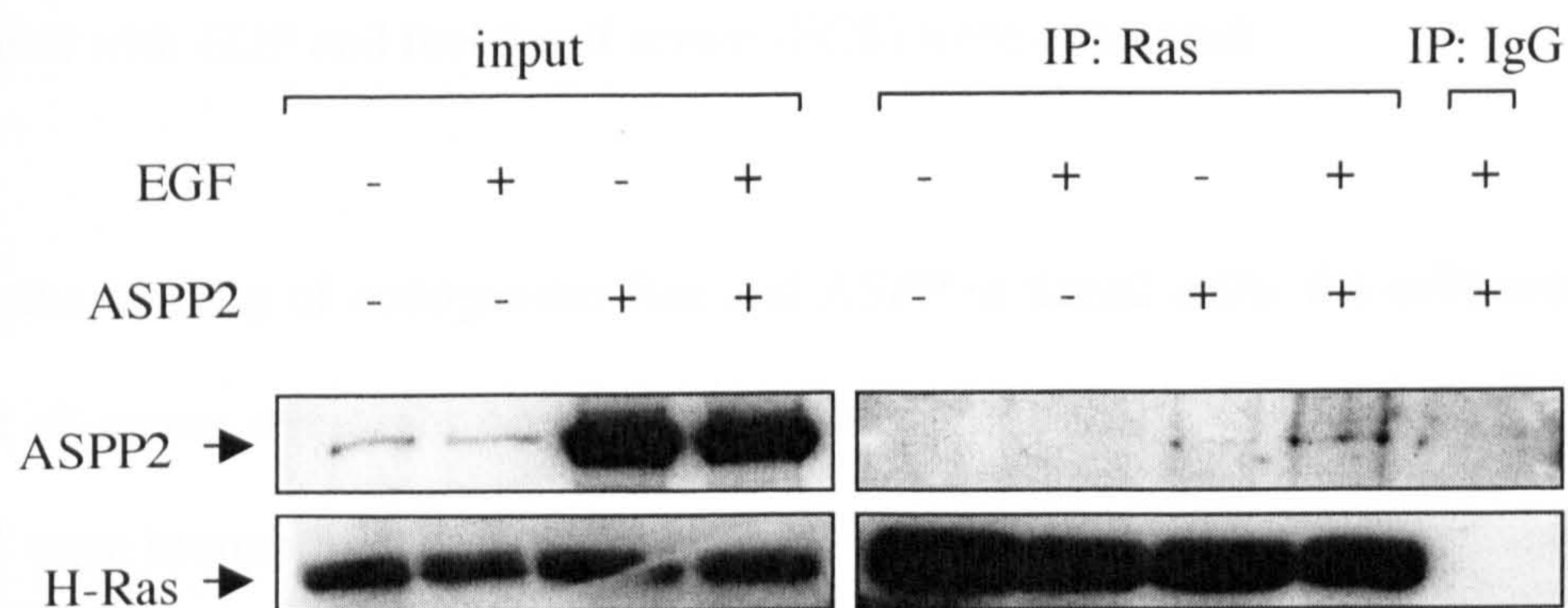


Figure 3.6 *Endogenous ras binds induced ASPP2 after EGF stimulation.* An ASPP2 inducible cells line made in U2OS cells with a tet-off system was used. The induced and uninduced cells were either grown in low percentage serum for 20 hours or grown in 10% FCS and EGF. The lysates were immunoprecipitated with anti-ras antibody (238) or as a control with a non-specific IgG antibody. A western blot was performed on the immunoprecipitates; the presence of ASPP2 detected with the ASPP2-specific 5410 antibody and H-Ras with the RO2120 antibody.

3.2.6.2. Stimulated endogenous Ras binds endogenous ASPP1 and ASPP2

To test whether endogenous Ras can bind endogenous ASPP proteins, Saos2 cells were used to immunoprecipitate endogenous ASPP1 and ASPP2. Since Ras in its active form has a higher affinity for ASPP than in its non-active form, starved cells and those stimulated with EGF and foetal calf serum (FCS) were compared.

To test the binding of endogenous Ras and ASPP in Saos2 cells, the cells were either starved of serum overnight or stimulated with EGF and FCS overnight. ASPP1 and ASPP2 were immunoprecipitated with ASPP-specific antibodies. Endogenous Ras was detected by Western Blot and shown to have a higher affinity to ASPP1 and ASPP2 when stimulated with EGF and FCS (figure 3.7). ASPP1 bound more strongly to active Ras in these cells compared to ASPP2.

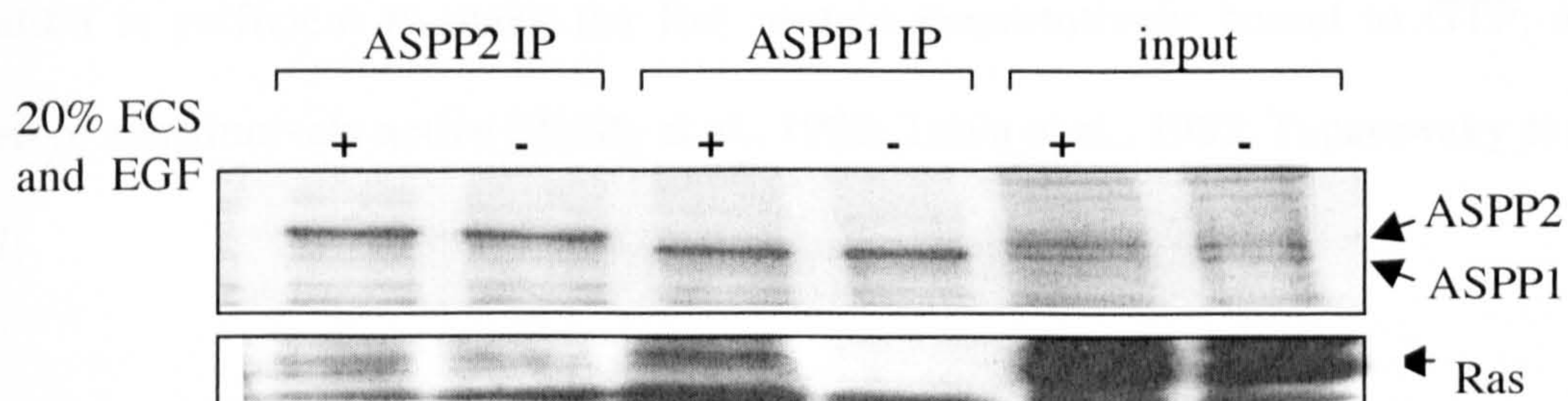


Figure 3.7 *Endogenous ras binds endogenous ASPP1 and ASPP2 after EGF stimulation.* Saos2 cells were either starved overnight in 0.5% FCS or grown in 20% FCS and EGF overnight. Lysates were collected and immunoprecipitated with either the ASPP1 polyclonal antibody ASPP1.8 or the ASPP2 polyclonal antibody bp77. A Western blot was performed and the presence of Ras detected with the RO2120 antibody. The ASPP proteins were detected with LX054.1 that is known to cross-react with both ASPP1 and ASPP2.

3.2.7. Oncogenic H-RasV12 and K-RasV12 stimulate ASPP1 and ASPP2 activity

3.2.7.1. ASPP2 activity is stimulated by oncogenic H-RasV12 and K-RasV12

Given that ASPP1 and ASPP2 have been shown to bind stimulated Ras *in vivo*, the next step was to see whether Ras had any effect on their activity. Since Ras in its GTP form is more likely to bind ASPP1 both *in vitro* and *in vivo*, oncogenic Ras was used to test its effect on ASPP. A single point mutation of H-Ras and K-Ras in their codon 12 changes their sequence from amino acid glycine to amino acid valine. This point mutation is sufficient to make the Ras protein constitutively bound to GTP, and therefore constitutively active (Reddy et al., 1982; Tabin et al., 1982; Taparowsky et al., 1982).

ASPP2 has been shown previously to increase p53 transactivation activity specifically on pro-apoptotic promoters. To test whether oncogenic Ras had any effect on ASPP2, a transactivation assay was performed with the pro-apoptotic bax-luciferase p53 reporter. Saos2 cells lacking endogenous p53 were transfected with p53 and ASPP2, in the presence or absence of K-RasV12 (figure 3.8A). It was clear from the luciferase counts that K-RasV12 can increase ASPP2 stimulation of p53 by approximately 2.5 fold (figure 3.8B). This increase of p53 activity by K-RasV12 was due to ASPP2 activity as K-RasV12 did not have a significant effect on p53 in the absence of transfected ASPP2.

Although encoded by a different gene, H-Ras is very similar to K-Ras in its structure, function and signalling pathways. The effect of H-RasV12 on ASPP2 activity was therefore analyzed. A similar experiment to that described above was performed with H-RasV12 transfected instead of K-RasV12. As expected, H-RasV12 can increase ASPP2 activity to the same extent as K-RasV12 (figure 3.8E).

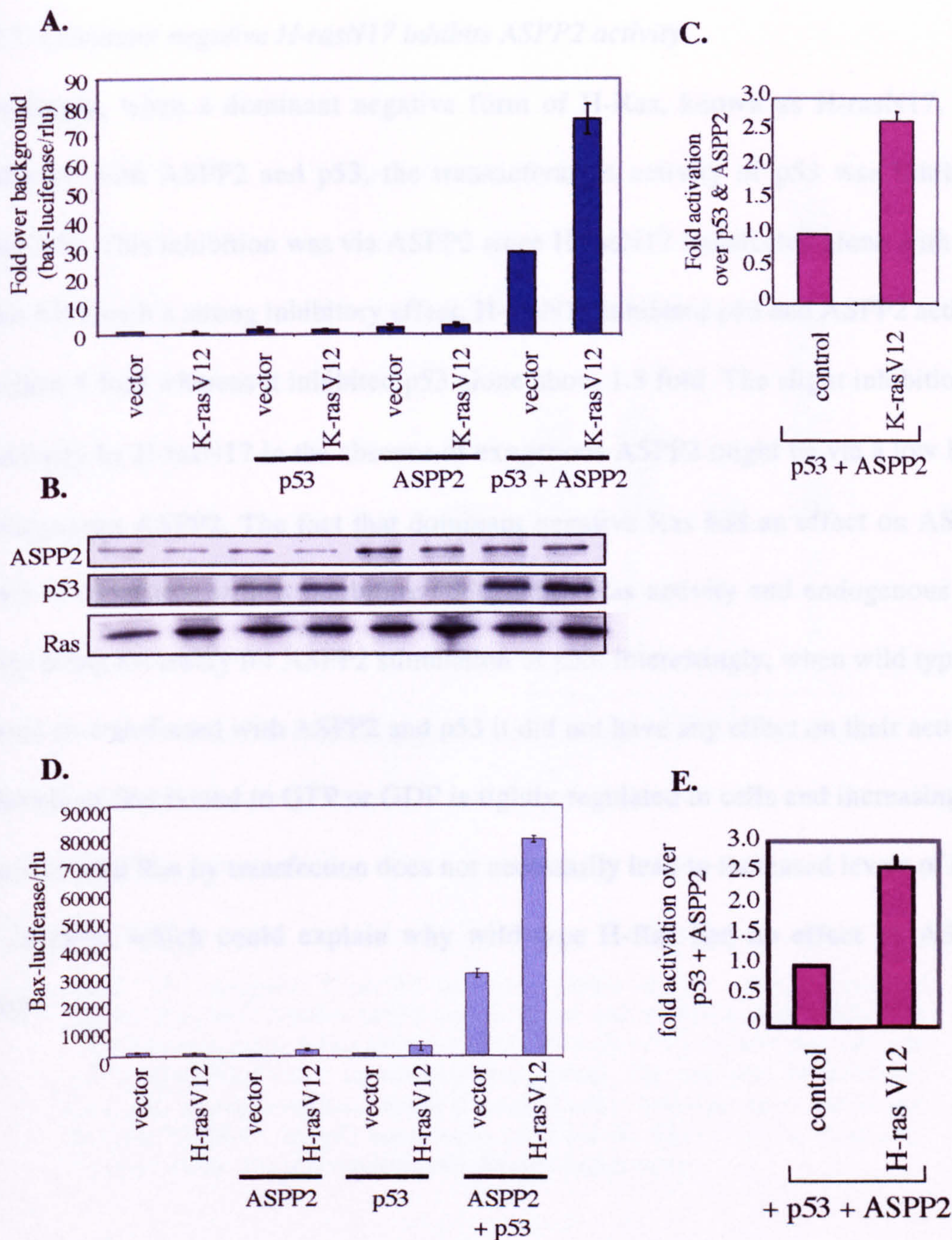


Figure 3.8 *H-rasV12 and K-rasV12 stimulate ASPP2 activity to the same extent.* (A). Saos2 cells were transfected with 50 ng p53, 4 μ g ASPP2 and 1.5 μ g K-rasV12 as indicated. All cells were co-transfected with bax-luciferase. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. The luciferase counts are shown. (B). An aliquot of luciferase samples from A were used for a western blot. ASPP2 was detected with the 5410 antibody, p53 with DO-1 and K-rasV12 with RO2120 antibody. (C). The fold increase of p53 and ASPP2 activity in the presence of K-rasV12 is shown. (D). Saos2 cells were transfected with 50 ng p53, 4 μ g ASPP2 and 1.5 μ g of H-rasV12 as indicated. All cells were co-transfected with bax-luciferase. (E). The fold increase of p53 and ASPP2 luciferase activity in the presence of H-rasV12 is shown.

3.2.7.2. Dominant negative H-rasN17 inhibits ASPP2 activity

Interestingly, when a dominant negative form of H-Ras, known as H-rasN17, was transfected with ASPP2 and p53, the transactivation activity of p53 was inhibited (figure 3.9). This inhibition was via ASPP2 since H-rasN17 transfected alone with p53 did not have such a strong inhibitory effect: H-rasN17 inhibited p53 and ASPP2 activity more than 4 fold whereas it inhibited p53 alone about 1.5 fold. The slight inhibition of p53 activity by H-rasN17 in the absence of exogenous ASPP2 might be via a low level of endogenous ASPP2. The fact that dominant negative Ras had an effect on ASPP2 activity is consistent with it inhibiting endogenous Ras activity and endogenous Ras activity being necessary for ASPP2 stimulation of p53. Interestingly, when wild type H-Ras was co-transfected with ASPP2 and p53 it did not have any effect on their activity. The levels of Ras bound to GTP or GDP is tightly regulated in cells and increasing the amount of total Ras by transfection does not necessarily lead to increased levels of Ras-GTP in cells, which could explain why wild-type H-Ras had no effect on ASPP2 activity.

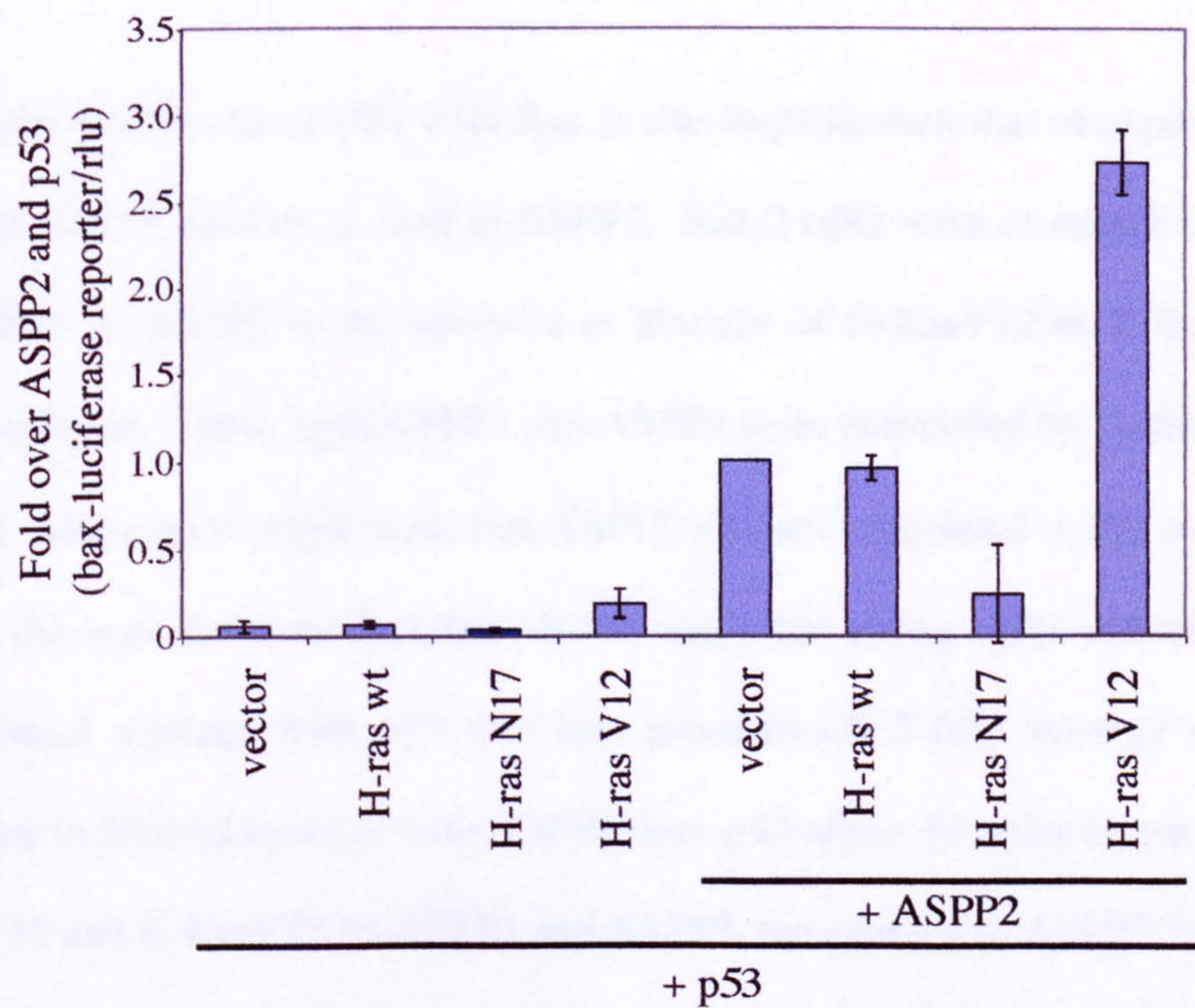


Figure 3.9 *Oncogenic H-rasV12 increases ASPP2 activity whereas dominant negative H-RasN17 inhibits ASPP2 activity.* Saos2 cells were transfected with 4 μ g ASPP2, 50 ng p53 and 1.5 μ g H-ras wt, H-rasN17 or H-rasV12 as indicated. All cells were co-transfected with 1 μ g bax-luciferase reporter. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. The value of ASPP2 and p53 are taken as 1.0 to see the effect of ras on its activity. The mean values were derived from two different experiments.

3.2.7.3. Both ASPP1 and ASPP2 are stimulated to the same extent by H-RasV12 and K-RasV12

Since both ASPP1 and ASPP2 bind Ras, it was hypothesised that oncogenic Ras could stimulate ASPP1 activity as well as ASPP2. Saos2 cells were co-transfected with p53 and ASPP1 or ASPP2 in the presence or absence of H-RasV12 or K-RasV12. As is shown in figure 3.10A, both ASPP1 and ASPP2 were stimulated by H-RasV12 and K-RasV12. Although it might seem that ASPP1 was not stimulated to the same extent as ASPP2, this was due to the fact that ASPP1 was not as strong a p53-activator as ASPP2 so the basal synergy with p53 was less pronounced: 5-fold synergy with ASPP1 compared to 20-fold synergy with ASPP2 over p53 alone. In order to see the effect of H-RasV12 and K-RasV12 on ASPP1 and ASPP2, the synergy of ASPP1/2 and p53 was taken to be 1 and the fold increase by oncogenic Ras calculated (figure 3.10C). Both H-RasV12 and K-RasV12 stimulated ASPP1 and ASPP2 activity to the same extent, namely 2.5-fold.

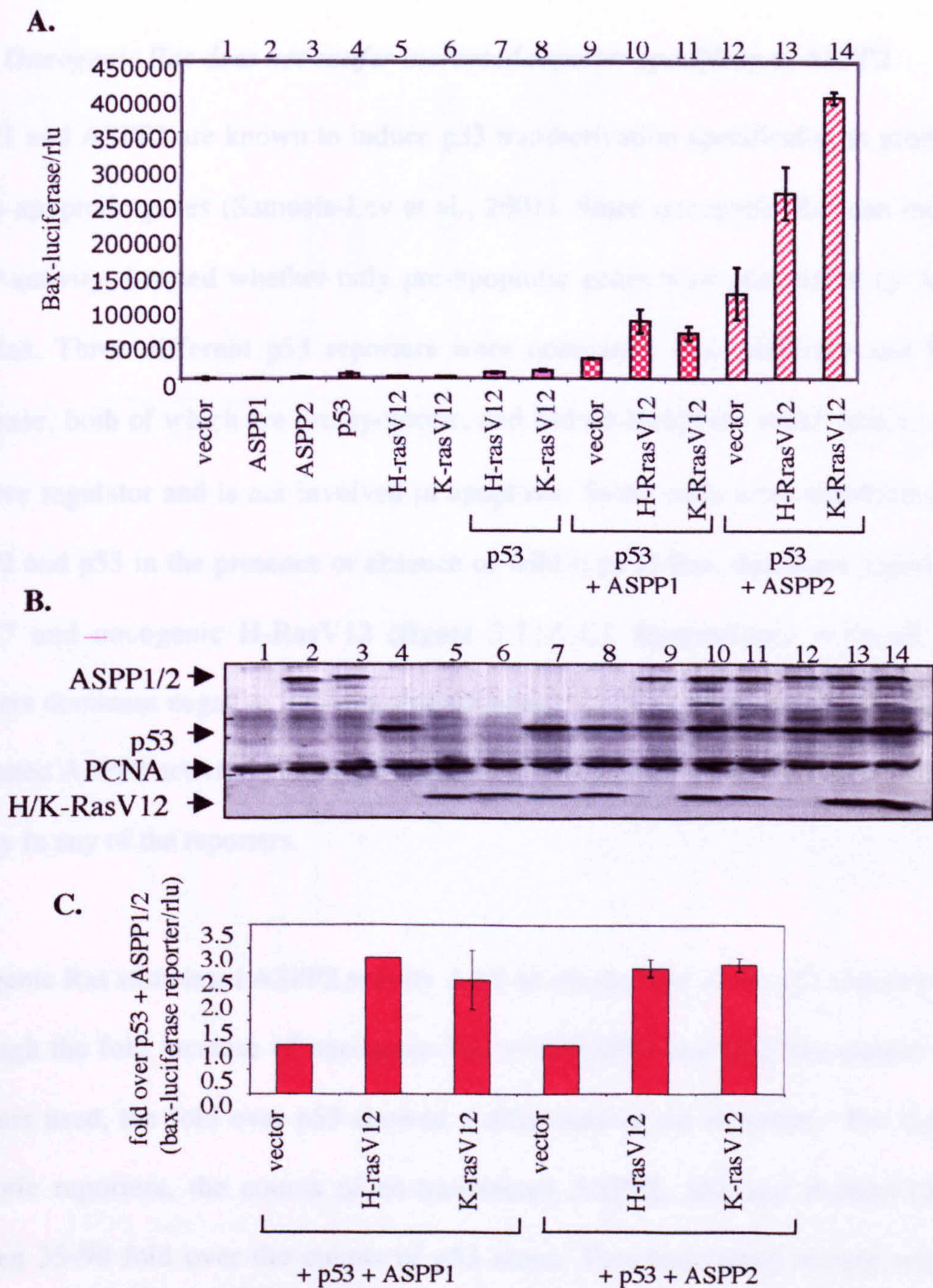


Figure 3.10 ASPP1 and ASPP2 are activated to a similar extent by H-rasV12 and K-rasV12. (A). Saos2 cells were transfected with 50 ng p53 and 4 μ g of ASPP1 or ASPP2. 1.5 μ g of H-rasV12 or K-rasV12 was co-transfected where indicated. All samples were co-transfected with bax-luciferase. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. (B). After 20 hours cell lysates were prepared. An aliquot was resolved on 10% SDS-PAGE gel for western blotting for ASPP1 and ASPP2 with the V5 antibody, p53 with the DO-1 antibody and transfected H-rasV12 and K-rasV12 with the HA antibody. PCNA was used as a loading control (PC-10 antibody). The numbers above the gel are equivalent to the numbered samples shown in A. (C). The left part of the graph shows the fold increase of ASPP1 and p53 in the presence of H-rasV12 and K-rasV12. The right part of the graph shows the fold increase of ASPP2 and p53 in the presence of H-rasV12 and K-rasV12. The mean values are derived from three independent experiments for ASPP1 and ASPP2.

3.2.8. Oncogenic Ras does not confer increased reporter specificity to ASPP2

ASPP1 and ASPP2 are known to induce p53 transactivation specifically on promoters of pro-apoptotic genes (Samuels-Lev et al., 2001). Since oncogenic Ras can increase ASPP activity, I tested whether only pro-apoptotic genes were stimulated by ASPP2 and Ras. Three different p53 reporters were compared: bax-luciferase and PIG3-luciferase, both of which are pro-apoptotic, and Mdm2-luciferase which acts as a p53 negative regulator and is not involved in apoptosis. Saos2 cells were transfected with ASPP2 and p53 in the presence or absence of wild-type H-Ras, dominant negative H-rasN17 and oncogenic H-RasV12 (figure 3.11A-C). Interestingly, with all three reporters dominant negative Ras was able to reduce ASPP2 activity and oncogenic Ras stimulated ASPP2 activity. Wild type H-Ras did not have any effect on ASPP2 and p53 activity in any of the reporters.

Oncogenic Ras stimulated ASPP2 activity 2-3 fold irrespective of the p53-reporter used. Although the fold increase of oncogenic Ras over ASPP2 and p53 was similar in all reporters used, the fold over p53 showed a difference in the reporters. For the pro-apoptotic reporters, the counts of co-transfected ASPP2, p53 and H-RasV12 was between 35-90 fold over the counts of p53 alone. This contrasted sharply with the Mdm2-reporter which had a mere 8-fold increase in the presence of ASPP2, p53 and H-RasV12 compared to p53 alone. Although the fold of ASPP2 stimulation by oncogenic Ras was always 2-3 fold, the intrinsic selectivity of ASPP2 for pro-apoptotic genes was retained (figure 3.11D). As in the other experiments, the stimulation of p53 by oncogenic Ras was probably via ASPP2 since p53 without co-transfected ASPP2 was not stimulated to the same extent by oncogenic Ras.

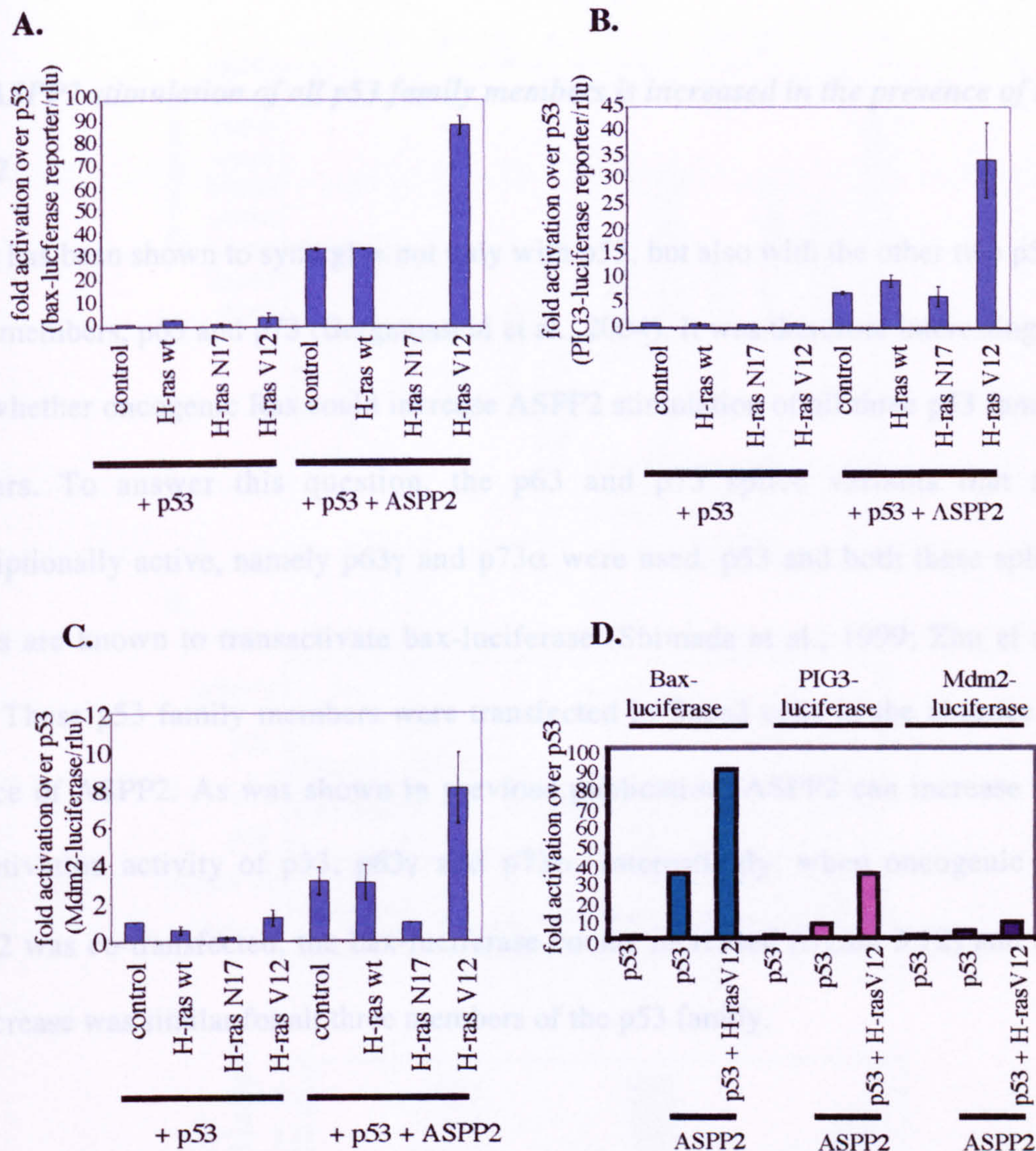


Figure 3.11 *H-rasN17* and *H-rasV12* do not confer additional reporter specificity to ASPP2. Saos2 cells were transfected with 4 μ g ASPP2 and 50 ng p53 in the presence of 1.5 μ g of either H-ras wt, H-RasN17 or H-rasV12 as indicated. The cells were co-transfected with bax-luciferase (A), PIG3-luciferase (B) or Mdm2-luciferase (C) reporters. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. The values of p53 alone were taken as one and all other values are shown as a fold activation over p53 alone. (D) The values from graphs A, B, and C were consolidated on one graph. The value of the transactivation p53 alone is set as one. The fold activation over p53 is shown when ASPP2 is co-transfected alone or with H-rasV12.

3.2.9. ASPP2 stimulation of all p53 family members is increased in the presence of H-RasV12

ASPP2 has been shown to synergize not only with p53, but also with the other two p53-family members, p63 and p73 (Bergamaschi et al., 2004). It was therefore interesting to know whether oncogenic Ras could increase ASPP2 stimulation of all three p53 family members. To answer this question, the p63 and p73 splice variants that are transcriptionally active, namely p63 γ and p73 α were used. p53 and both these splice variants are known to transactivate bax-luciferase (Shimada et al., 1999; Zhu et al., 1998). These p53 family members were transfected in Saos2 cells in the absence or presence of ASPP2. As was shown in previous publication, ASPP2 can increase the transactivation activity of p53, p63 γ and p73 α . Interestingly, when oncogenic H-RasV12 was co-transfected, the bax-luciferase counts increased (figure 3.12) and the fold increase was similar for all three members of the p53 family.

It is known that oncogenic Ras can stimulate Arf resulting in Mdm2 being unable to negatively regulate p53 (Palmero et al., 1998; Pomerantz et al., 1998). However, p63 and p73 are not believed to be regulated by Mdm2 nor Arf. The fact that H-RasV12 can increase ASPP2 stimulation of all three p53 family members indicates that its effect is probably independent of Arf.

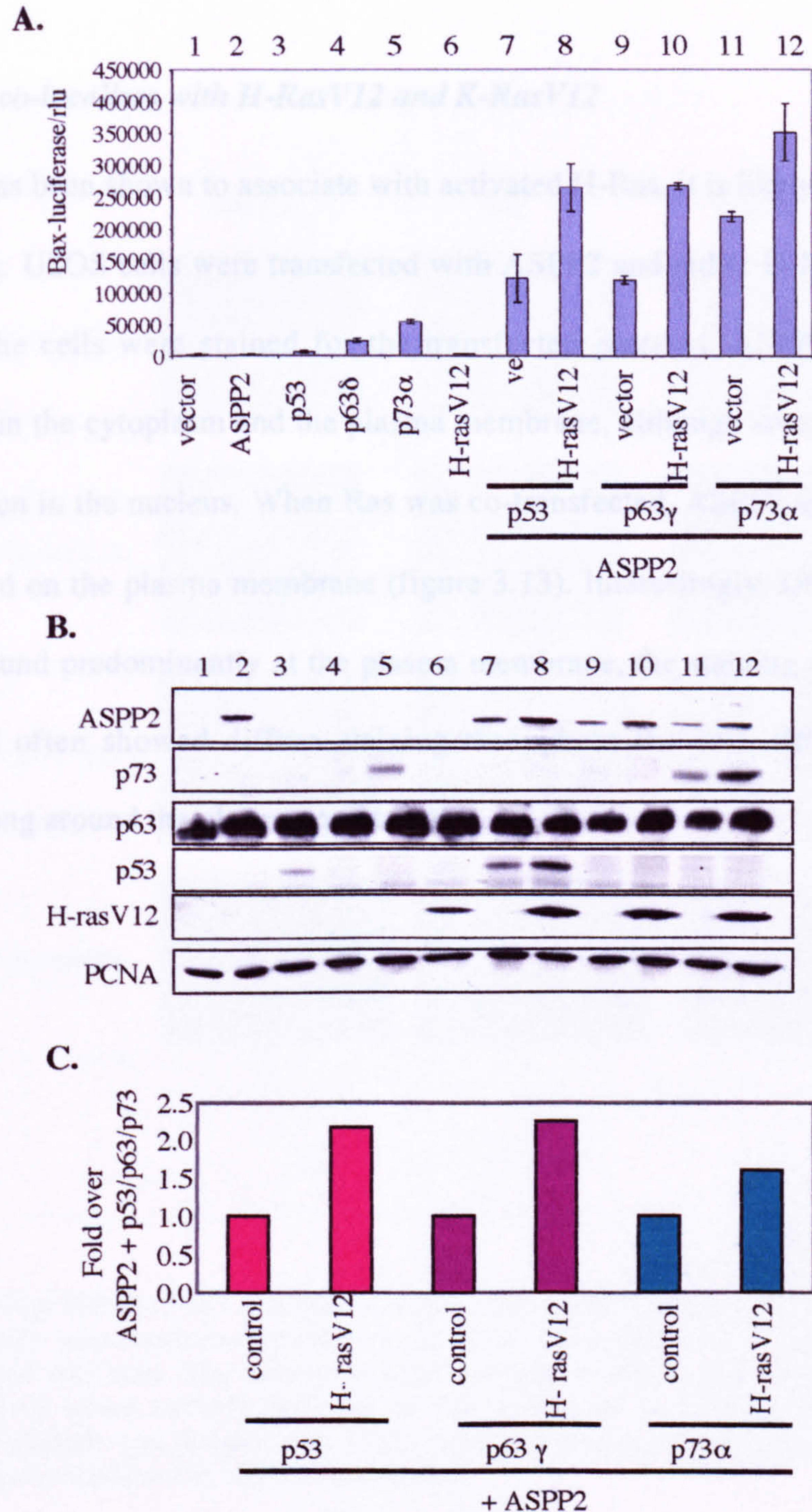
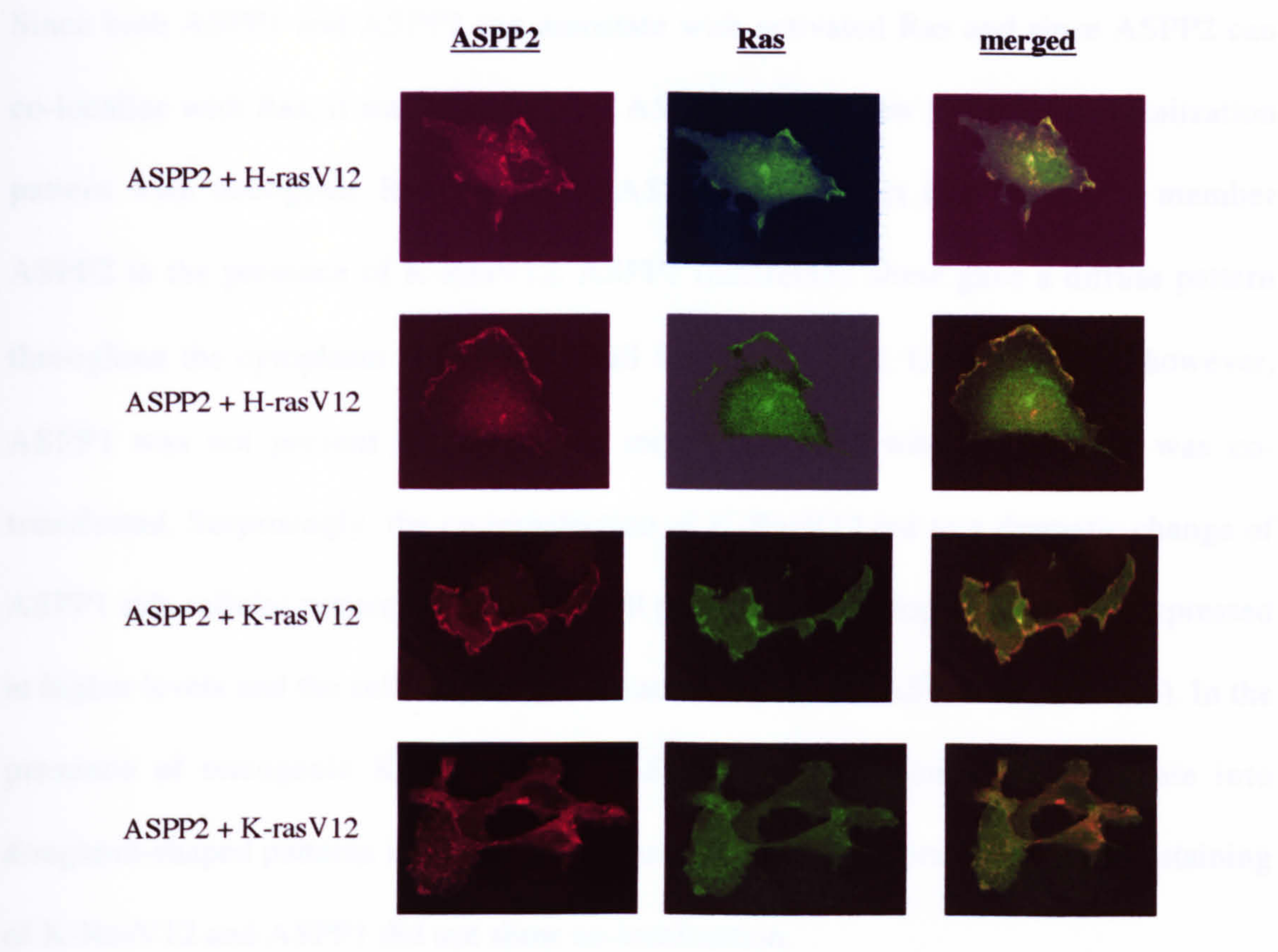


Figure 3.12 *Oncogenic H-rasV12 increases ASPP2 stimulation of all p53 family members.* (A). Saos2 cells were transfected with ASPP2 and co-transfected with p53, p63 γ or p73 α in the presence or absence of H-rasV12. All samples were co-transfected with bax-luciferase. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. (B). The samples shown in A were resolved on an SDS-PAGE gel and western blotting was performed. The numbers on the top of the gel represents the numbered sample shown in A. The blot was probed with the V5 antibody to detect ASPP2, the HA antibody to detect p73 and H-rasV12, the 4A4 antibody to detect p63, DO-1 for p53 and PC-10 for PCNA. (C). The graph shows the fold increase over ASPP2 and the p53-family members by H-rasV12.

3.2.10. ASPP2 co-localizes with H-RasV12 and K-RasV12

Since ASPP2 has been shown to associate with activated H-Ras, it is likely that they co-localize in cells. U2OS cells were transfected with ASPP2 and either K-RasV12 or H-RasV12, and the cells were stained for the transfected proteins. ASPP2 was found predominantly in the cytoplasm and the plasma membrane, although some protein was occasionally seen in the nucleus. When Ras was co-transfected, ASPP2 and oncogenic Ras co-localized on the plasma membrane (figure 3.13). Interestingly, although Ras is known to be found predominantly at the plasma membrane, the staining of H-RasV12 and K-RasV12 often showed diffuse staining throughout the cell, although it was particularly strong around the plasma membrane.

3.2.11. *H-rasV12* and *K-rasV12* co-localize with ASPP2 on the cell membrane



The ASPP2 and Ras co-localize on the cell membrane in cells transfected with ASPP2 and H-rasV12 or ASPP2 and K-rasV12.

Figure 3.13 *H-rasV12* and *K-rasV12* co-localize with ASPP2 on the cell membrane. U2OS cells were transfected with ASPP2 and H-rasV12 or ASPP2 and K-rasV12 (as shown on left lane). The cells were fixed and double-stained with the ASPP2 monoclonal mouse antibody 5410 and the Ras monoclonal rat antibody 259. The ASPP2 antibody was detected with TRITC label and the ras with FITC label. The staining was visualized by confocal microscopy.

3.2.11. K-RasV12 changes the sub-cellular localization of ASPP1

Since both ASPP1 and ASPP2 can associate with activated Ras and since ASPP2 can co-localize with Ras, it was expected that ASPP1 would show a similar co-localization pattern with oncogenic Ras. However, ASPP1 did not act like its family member ASPP2 in the presence of K-RasV12. ASPP1 transfected alone gave a diffuse pattern throughout the cytoplasm with some small dots throughout. Unlike ASPP2, however, ASPP1 was not present in the plasma membrane even when K-RasV12 was co-transfected. Surprisingly, the co-transfection of K-RasV12 led to a dramatic change of ASPP1 sub-cellular pattern. ASPP1 was still present in the cytoplasm but was expressed at higher levels and the cells stained much more brightly for ASPP1 (figure 3.14). In the presence of oncogenic K-RasV12, the ASPP1 proteins seemed to aggregate into doughnut-shaped patterns in the cytoplasm and perinuclear region. Merging the staining of K-RasV12 and ASPP1 did not show co-localization.

This result was unexpected as ASPP1 and ASPP2 showed no difference in their activity. Both could bind activated Ras and both are activated to the same extent by H-RasV12 and K-RasV12. There is little known in the literature about differences in the two proteins; the difference in sub-cellular localization after oncogenic Ras co-transfection may be due to unknown functional differences.

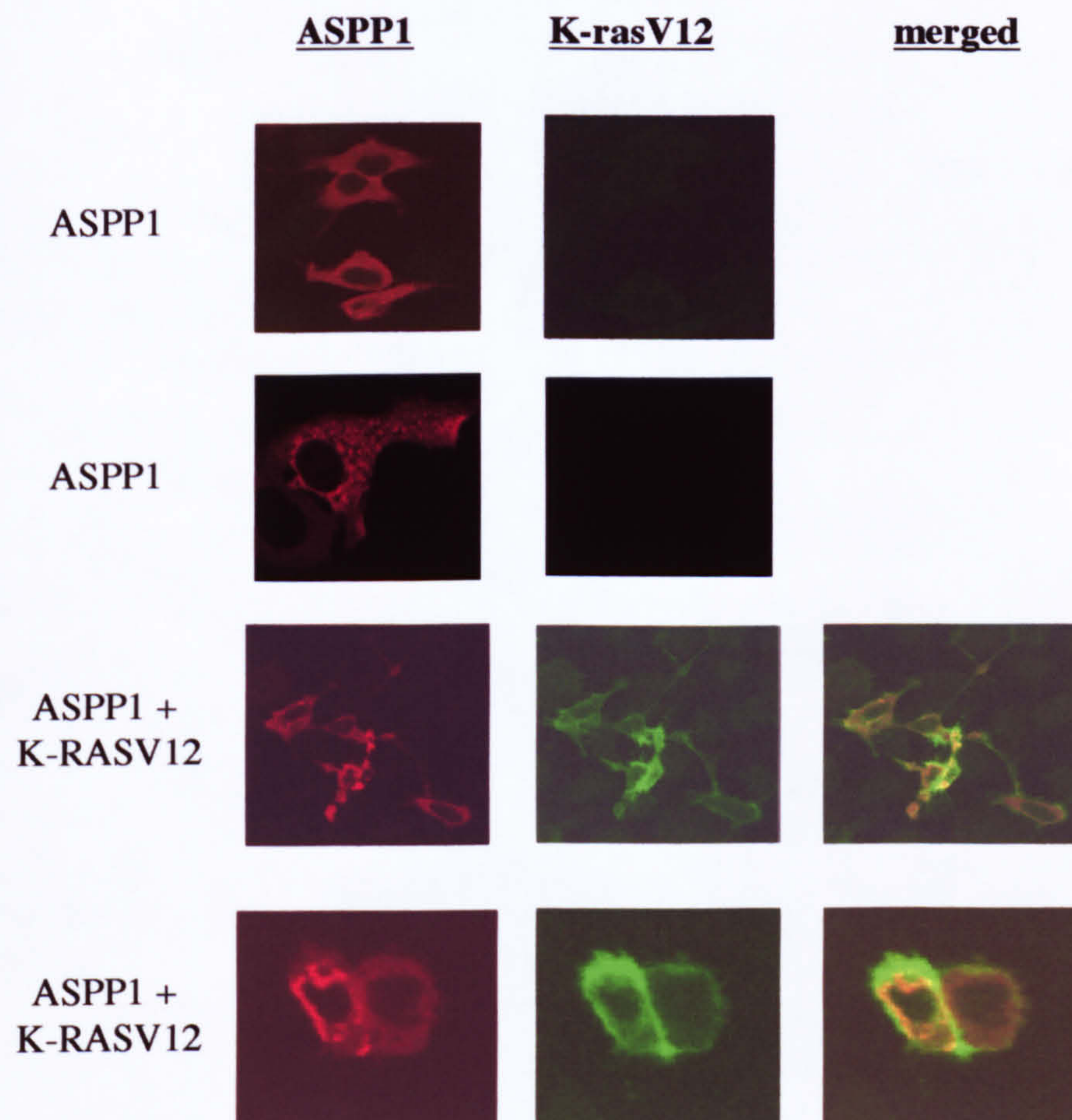


Figure 3.14 *ASPP1 sub-cellular localization changes in the presence of K-rasV12.* U2OS cells were transfected with ASPP1 alone or ASPP1 and K-rasV12 (as shown on left lane). The cells were fixed and double-stained with the V5 monoclonal mouse antibody against ASPP1 and the Ras monoclonal rat antibody 259. ASPP1 was detected with TRITC and ras with FITC. The staining was visualized by confocal microscopy.

3.2.12. Oncogenic Ras can transactivate pro-apoptotic genes via endogenous p53 and ASPP

So far, the effect of oncogenic Ras on pro-apoptotic reporters has been demonstrated using exogenous ASPP and p53. To investigate whether oncogenic Ras can stimulate endogenous ASPP and p53 activity, a different system was used. MCF7 and U2OS cells both contain wild-type p53 and are known to have reasonably high levels of endogenous ASPP1 and ASPP2. Those cells were transfected with H-RasV12 and K-RasV12 in the presence of bax-luciferase in the case of MCF7 cells and with PIG3-luciferase in the case of U2OS cells (figure 3.15A and B, respectively). Both oncogenic Ras genes were able to transactivate the p53 reporters. Interestingly, in the presence of published anti-sense ASPP1 and anti-sense ASPP2 plasmids, the transactivation activity of oncogenic Ras was strongly inhibited, suggesting that it was activating the reporters via the ASPP1 and ASPP2 pathways. To confirm this, the p53 viral inhibitor E6 was co-transfected and it too significantly reduced the oncogenic Ras transactivation activity. It has been reported that iASPP is an inhibitor of p53 by preventing its activation by ASPP1 and ASPP2 (Bergamaschi et al., 2003b). To further test whether the oncogenic Ras could transactivate the pro-apoptotic reporters via ASPP1 and ASPP2, the inhibitor iASPP was co-transfected and, as expected, it too inhibited the transactivation activity of H-RasV12 and K-RasV12.

The anti-sense ASPP1 and anti-sense ASPP2 plasmids have been shown to specifically inhibit ASPP1 and ASPP2, respectively (Samuels-Lev et al., 2001). Anti-sense prevents the translation of the mRNA so it is expected that the ASPP1 and ASPP2 expression levels would be decreased in the presence of anti-sense ASPP1 and anti-sense ASPP2. To test this, the lysates from the transactivation experiment were used. Since the

transactivation lysis buffer is made with a weak detergent, both the supernatant and the resuspended pellet were analyzed by Western blot. Figure 3.15C showed that no difference in ASPP1 and ASPP2 expression was detected after anti-sense transfection. This was most probably due to the fact that only a small percentage of the cells were transfected with anti-sense plasmids and the Western blot showed the ASPP levels from the total amount of cells.

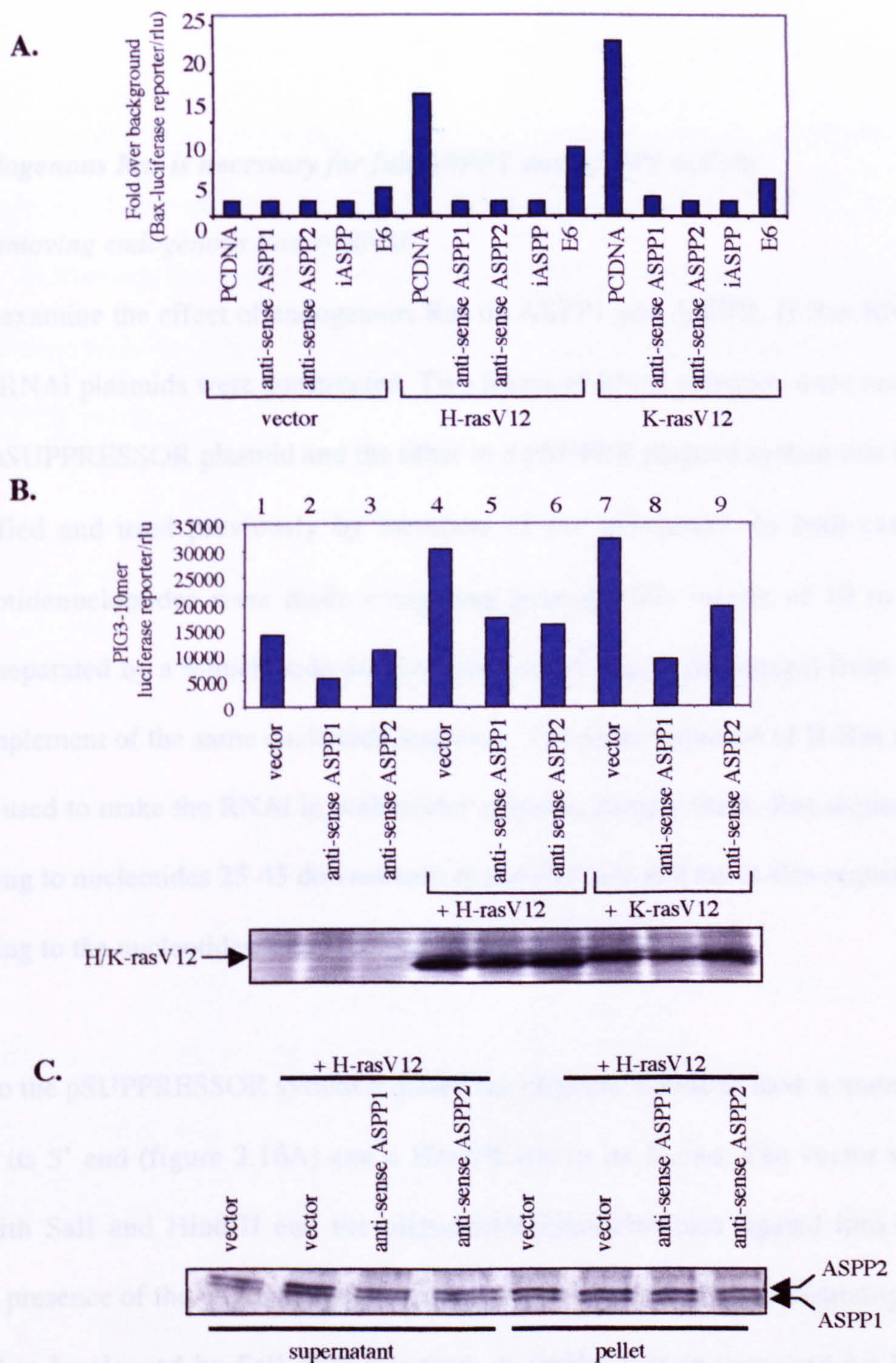


Figure 3.15 *H-rasV12* and *K-rasV12* activate endogenous *ASPP1* and *ASPP2*. (A) MCF7 cells were transfected with 6 μ g anti-sense *ASPP1* or anti-sense *ASPP2*, in the presence or absence of 1.5 μ g of *H-rasV12* or *K-rasV12*. All cells were co-transfected with bax-luciferase reporter. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. (B) A similar experiment to A was performed in U2OS cells with PIG3-luciferase reporter. The lysate was used for Western blotting and the presence of transfected *H-rasV12* and *K-rasV12* was detected with the HA antibody. (C) Lysates 1 and 4-6 from B were taken and the supernatant separated from the pellet. The pellet was further resuspended in 1x RIPA buffer. An equal amount of protein was loaded onto a 8% SDS-PAGE gel and western blotting was performed to detect *ASPP1* and *ASPP2* with the antibody LX054.1 which is known to cross-react with both proteins.

3.2.13. Endogenous Ras is necessary for full ASPP1 and ASPP2 activity

3.2.13.1. Removing endogenous Ras by RNAi

In order to examine the effect of endogenous Ras on ASPP1 and ASPP2, H-Ras RNAi and K-Ras RNAi plasmids were constructed. Two forms of RNAi plasmids were made, one in the pSUPPRESSOR plasmid and the other in a pSUPER plasmid system that has been modified and used previously by members of our laboratory. In both cases, oligonucleotidenucleotides were made containing gene-specific inserts of 19 to 20 nucleotide separated by a 9-nucleotide non-complementary spacer (ttcaagaga) from the reverse complement of the same nucleotide sequence. The same sequence of H-Ras and K-Ras was used to make the RNAi in both vector systems, namely the K-Ras sequence corresponding to nucleotides 25-43 downstream of the start site and the H-Ras sequence corresponding to the nucleotides 299-316 downstream of the start site.

Cloning into the pSUPPRESSOR system required the oligonucleotide to have a mutated SalI site in its 5' end (figure 3.16A) and a HindIII site in its 3' end. The vector was digested with SalI and HindIII and the oligonucleotidenucleotides ligated into the vector. The presence of the oligonucleotidenucleotides was identified by the inability of the plasmid to be cleaved by SalI after insertion. A similar system was used for Ras RNAi cloning into the pSUPER system with a BglII mutated site in the 5' end of the oligonucleotide and a HindIII site in the 3' end of the oligonucleotide (figure 3.16B).

The specificity of the two RNAi systems were tested by co-transfecting tagged HA-H-RasV12 and HA-K-RasV12 into cells, with the RNAi constructs (figure 3.16B). In the pSUPPRESSOR system, these oligonucleotides did not seem to have specificity as both H-RasV12 and K-RasV12 expression was reduced in the presence of H-Ras RNAi or

K-Ras RNAi. The pSUPER system however was effective and specific: only the H-Ras pSUPER reduced H-Ras expression and only the K-Ras pSUPER reduced the K-Ras expression. Following these results, only the pSUPER system was used and all experiments done with Ras RNAi were done with the oligonucleotidenucleotides cloned into the pSUPER vector.

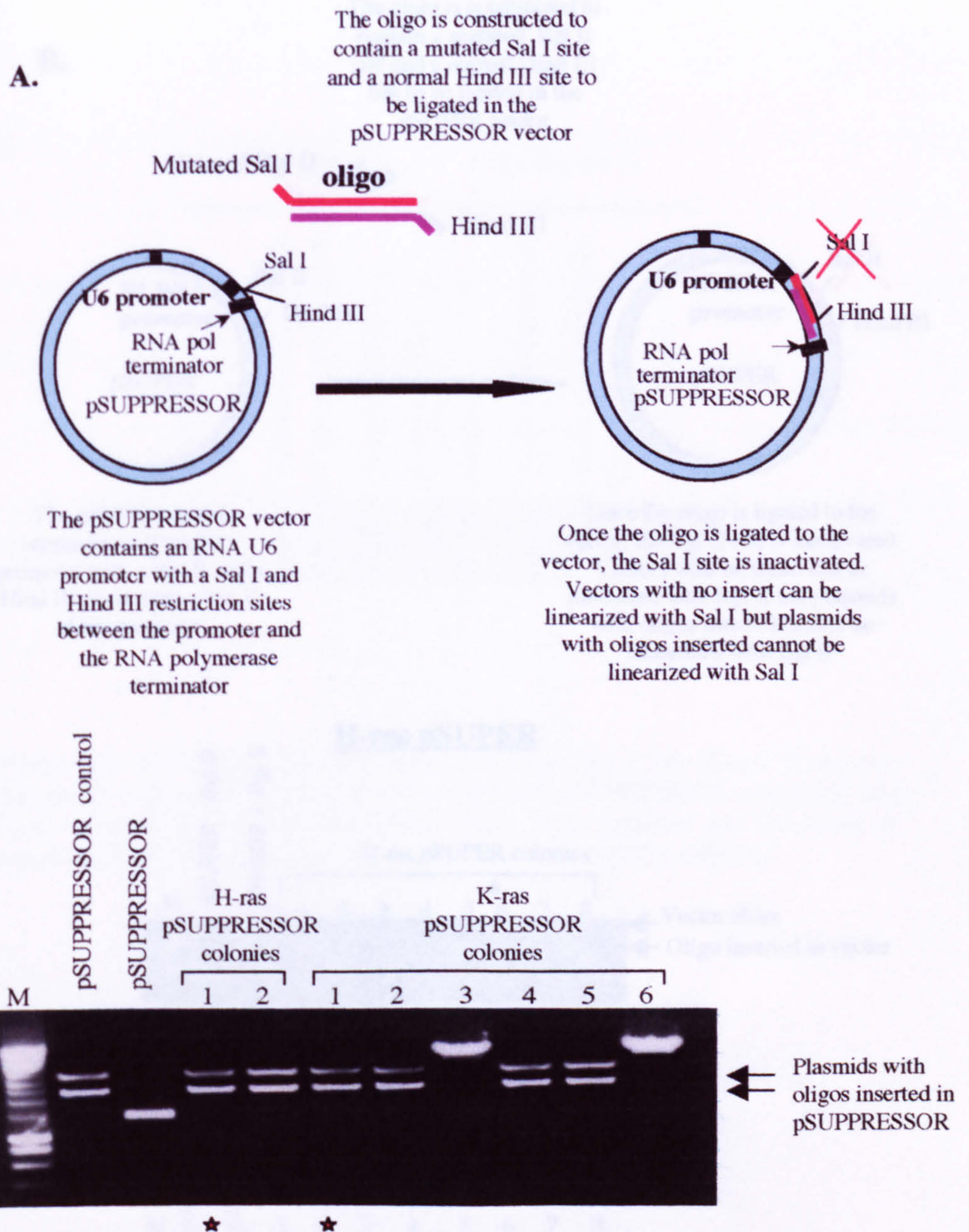
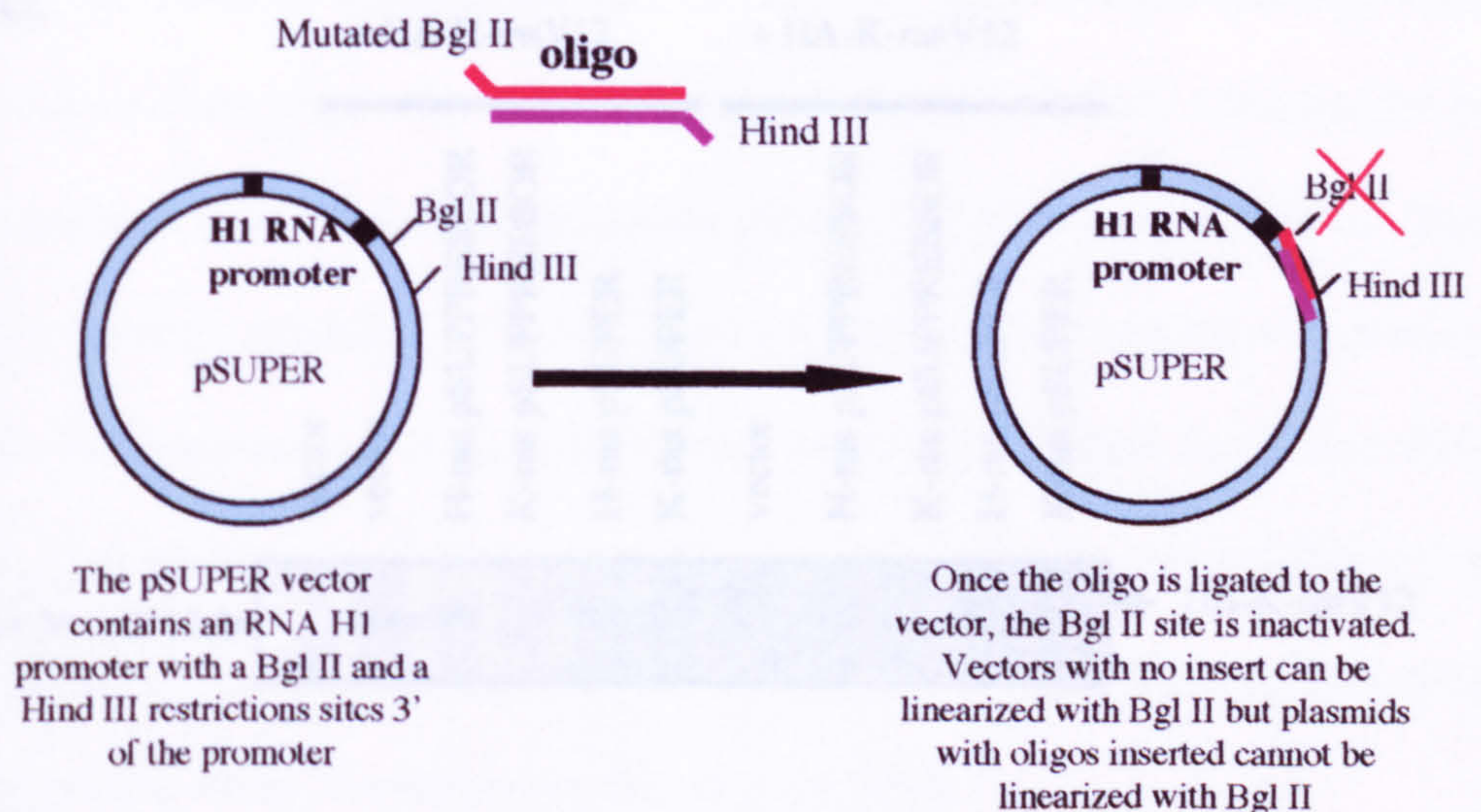


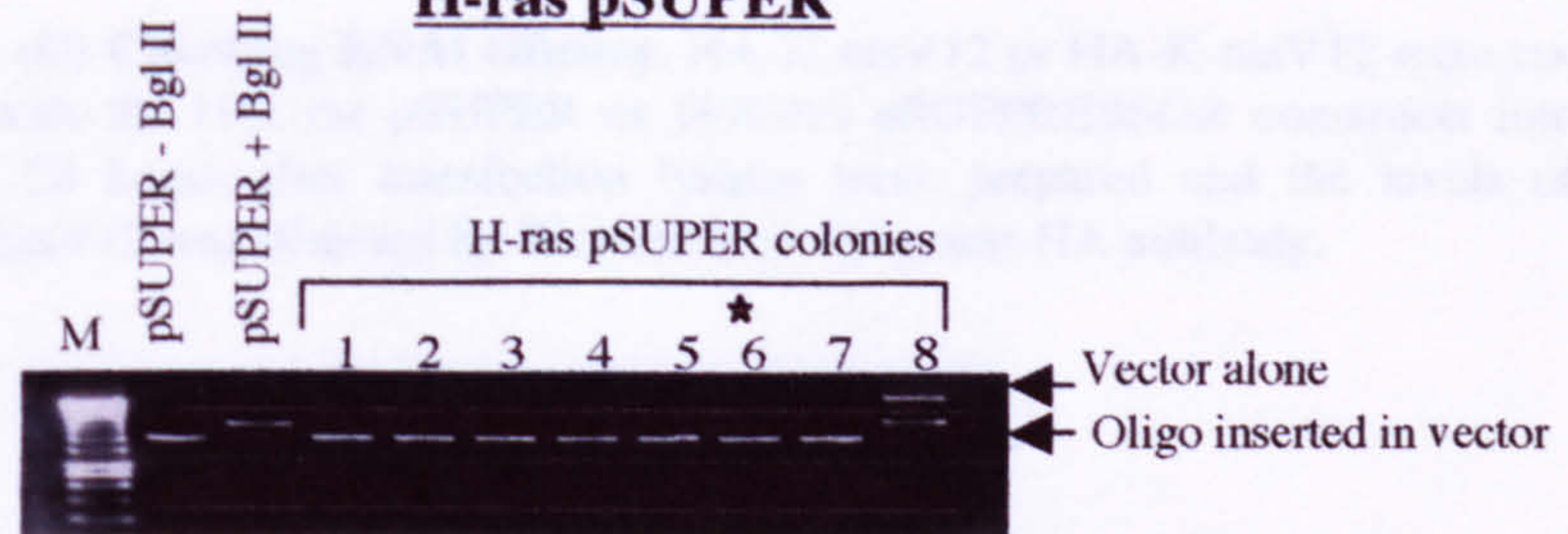
Figure 3.16 Construction of plasmids expressing *H-ras* RNAi and *K-ras* RNAi. (A) Cloning *ras* RNAi into pSUPPRESSOR vector. The sequence used to clone *K-ras* into pSUPPRESSOR was a 19-nucleotide sequence corresponding to nucleotides 25-43 downstream of the start site. Details of oligonucleotide sequences are given in Materials & Methods. The *H-ras* RNAi construct has a 20-nucleotide sequence corresponding to the nucleotides 299-316 downstream of the start site. The oligonucleotides contain a mutated *Sal*I site in their 5' end and a *Hind*III site in their 3' ends. The complementary oligonucleotides were first annealed and they were then ligated into the pSUPPRESSOR vector which had been digested with *Sal*I and *Hind*III. The resulting constructs were digested with *Sal*I. Those uncleaved by *Sal*I contained the insert. One of each construct was selected (marked with a star) and used for further assays. The first lane shows a modified pSUPPRESSOR (control) that can no longer be cleaved by *Sal*I. The second lane shows a negative control: the parent pSUPPRESSOR that can be cleaved by *Sal*I.

B.

The oligo is constructed to contain a mutated Bgl II site and a normal Hind III site to be ligated in the pSUPER vector



H-ras pSUPER



K-ras pSUPER

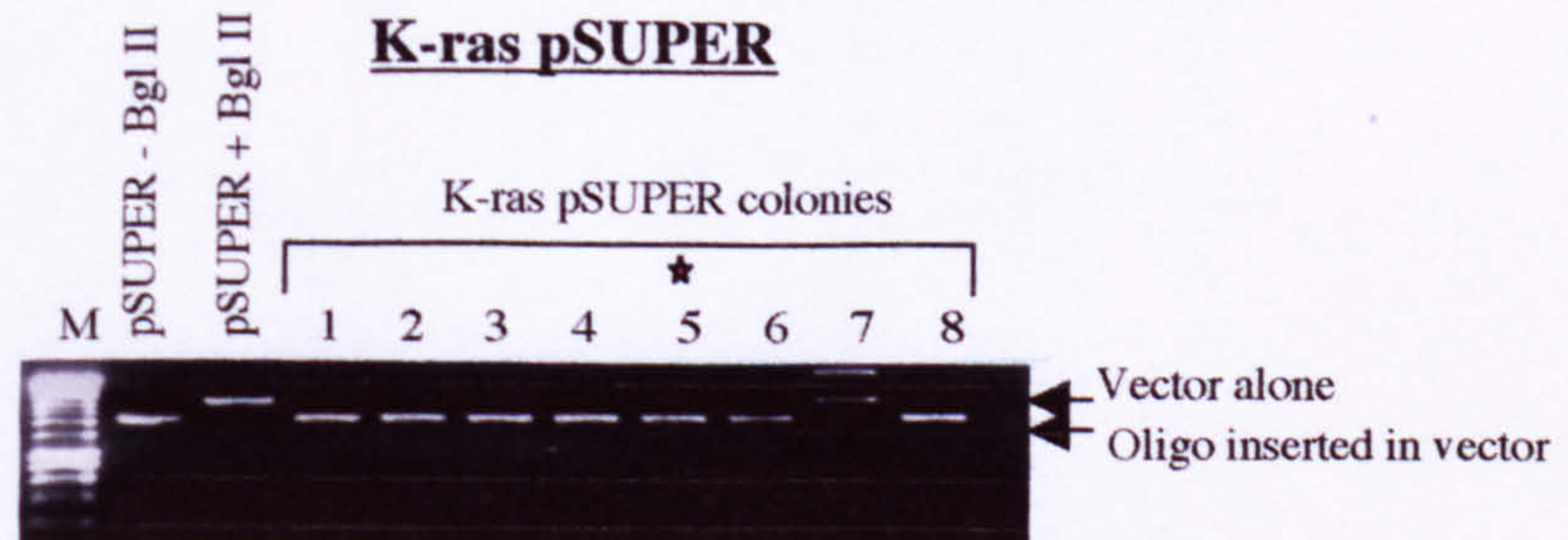


Figure 3.16. (B) Construction of pSUPER ras RNAi. The identical sequences of H-ras and K-ras were used as shown in A. The oligonucleotides contained a mutated Bgl II site in their 5' end and a Hind III site in their 3' ends. The complementary oligonucleotides were first annealed and they were then ligated into the pSUPER vector which had been digested with Bgl II and Hind III. The resulting constructs were digested with Bgl II. Those uncleaved by Bgl II contained the insert. One of each construct was selected (marked with a star) and used for further assays. The first lane shows a modified pSUPPRESSOR (control) that can no longer be cleaved by Sall. The second lane shows a negative control; the parent pSUPPRESSOR that can be cleaved by Sall.

3.2.13.2. Endogenous Ras is necessary for ASPP2 full transcriptional activity

To examine the effect of endogenous Ras on ASPP2 transcriptional activity, ASPP2 and p53 were co-transfected with the H/K-ras pSUPER or H/K-ras pSUPPRESSOR constructs into Saos2 cells. 20 hours after transfection lysates were prepared and the levels of transfected RasV12 was detected by Western blot using anti-HA antibody.

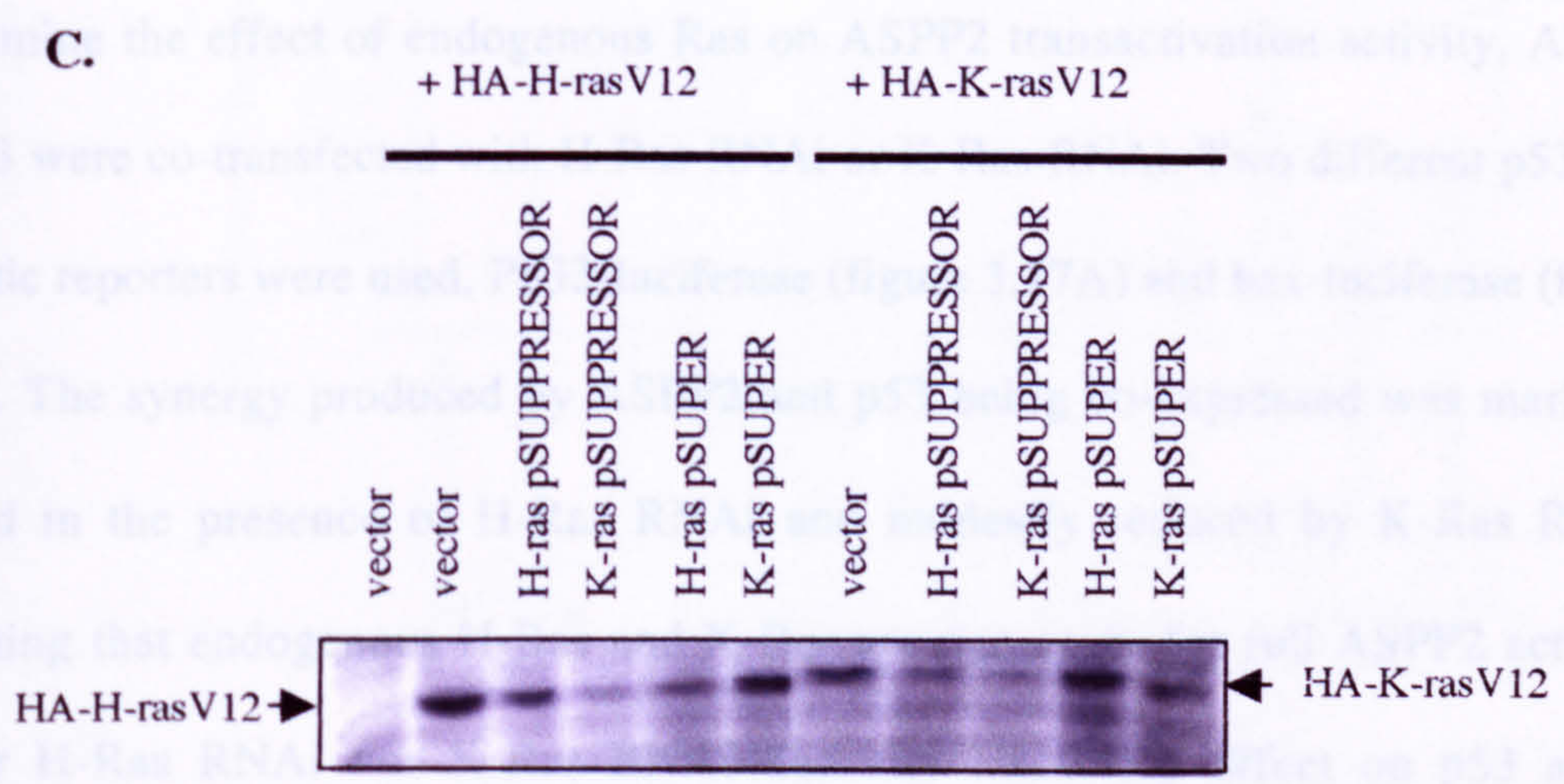


Figure 3.16. (C) Checking RNAi efficacy. HA-H-rasV12 or HA-K-rasV12 were co-transfected with the H/K-ras pSUPER or H/K-ras pSUPPRESSOR constructs into Saos2 cells. 20 hours after transfection lysates were prepared and the levels of transfected RasV12 was detected by Western blot using anti-HA antibody.

3.2.13.2. Endogenous Ras is necessary for ASPP2 full transactivation activity

To examine the effect of endogenous Ras on ASPP2 transactivation activity, ASPP2 and p53 were co-transfected with H-Ras RNAi or K-Ras RNAi. Two different p53 pro-apoptotic reporters were used, PIG3-luciferase (figure 3.17A) and bax-luciferase (figure 3.17B). The synergy produced by ASPP2 and p53 being co-expressed was markedly reduced in the presence of H-Ras RNAi and modestly reduced by K-Ras RNAi, suggesting that endogenous H-Ras and K-Ras are necessary for full ASPP2 activity. Neither H-Ras RNAi nor K-Ras RNAi had any inhibitory effect on p53 alone, suggesting that the effect of endogenous Ras is via ASPP2.

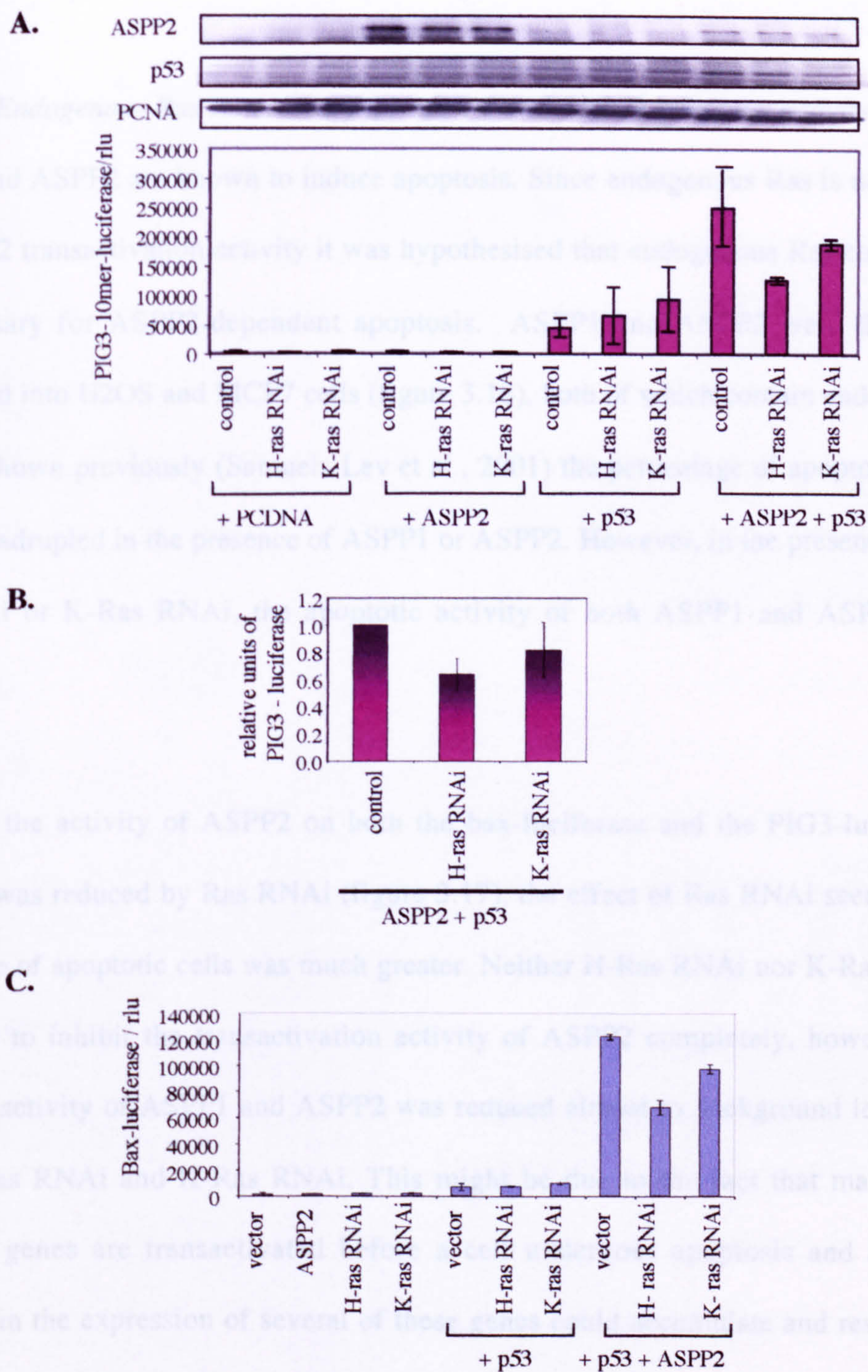


Figure 3.17 *Endogenous ras is necessary for ASPP2 full transactivation activity.* (A). Saos2 cells were transfected with 4 μ g ASPP2, 50 ng p53 and 4 μ g H-ras RNAi or K-ras RNAi as indicated. All cells were co-transfected with PIG3-10mer-luciferase reporter. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. The upper panel shows the western blot of each luciferase sample represented in the lower panel. ASPP2, p53 and PCNA were detected by Western blot using the antibodies 54.10, DO-1 and PC-10, respectively. (B). The mean values are derived from three independent experiments. p53 and ASPP2 are co-transfected with the PIG3-luciferase reporter in the presence or absence of H-ras RNAi and K-ras RNAi. The luciferase values with ASPP2 and p53 alone are taken as one. (C). Similar experiment to A with bax-luciferase as a reporter.

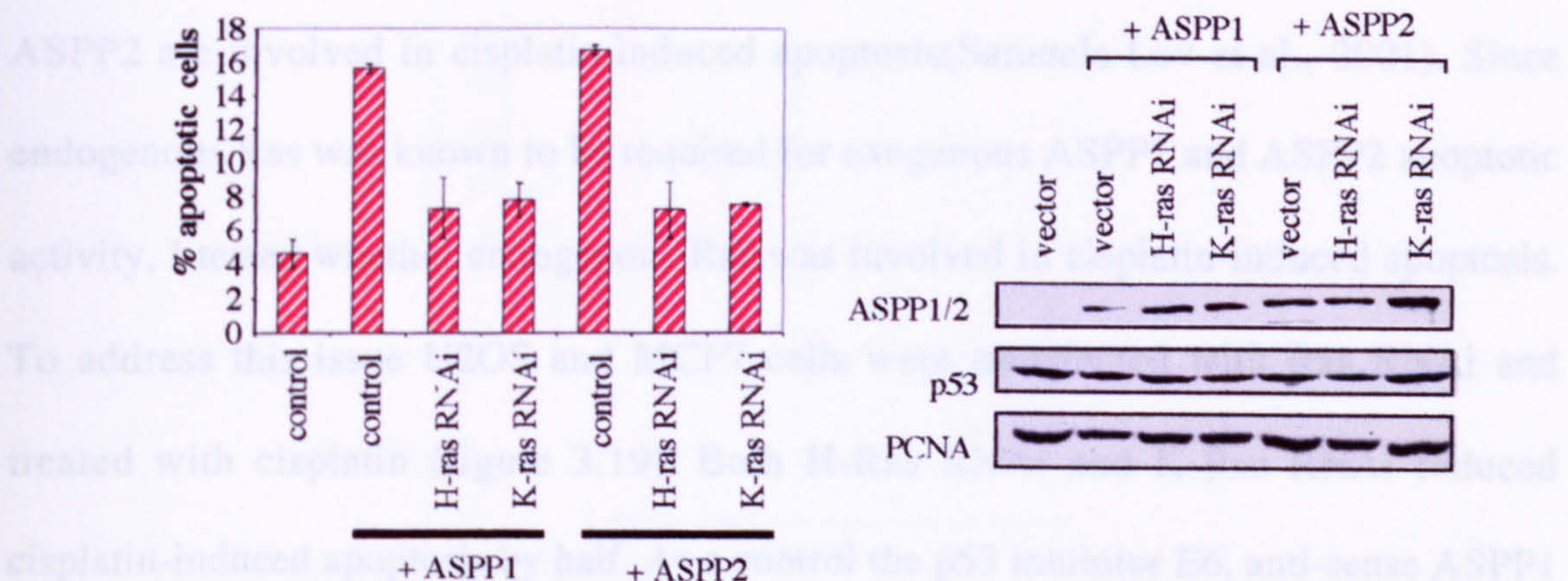
3.2.13.3. Endogenous Ras is necessary for ASPP1 and ASPP2 apoptotic activity

ASPP1 and ASPP2 are known to induce apoptosis. Since endogenous Ras is necessary for ASPP2 transactivation activity it was hypothesised that endogenous Ras could also be necessary for ASPP2-dependent apoptosis. ASPP1 and ASPP2 were therefore transfected into U2OS and MCF7 cells (figure 3.18), both of which contain endogenous p53. As shown previously (Samuels-Lev et al., 2001) the percentage of apoptotic cells almost quadrupled in the presence of ASPP1 or ASPP2. However, in the presence of H-Ras RNAi or K-Ras RNAi, the apoptotic activity of both ASPP1 and ASPP2 was inhibited.

Although the activity of ASPP2 on both the bax-luciferase and the PIG3-luciferase reporters was reduced by Ras RNAi (figure 3.17), the effect of Ras RNAi seen on the percentage of apoptotic cells was much greater. Neither H-Ras RNAi nor K-Ras RNAi were able to inhibit the transactivation activity of ASPP2 completely, however the apoptotic activity of ASPP1 and ASPP2 was reduced almost to background levels by both H-Ras RNAi and K-Ras RNAi. This might be due to the fact that many pro-apoptotic genes are transactivated before a cell undergoes apoptosis and a small reduction in the expression of several of these genes could accumulate and result in a significant overall reduction in apoptosis, as seen in figure 3.18.

3.2.13.4. Cisplatin-induced apoptosis requires endogenous H-Ras and K-Ras

Cisplatin is a known DNA damaging agent and can induce apoptosis via the p53 pathway (Prinschke et al., 1993; Fujiwara et al., 1994). It is known that ASPP1 and



To address the role of endogenous H-Ras and K-Ras in ASPP1- and ASPP2-mediated apoptosis, U2OS cells were transfected with 10 μ g ASPP1 or ASPP2 in the presence of 9 μ g H-ras RNAi or K-ras RNAi. Endogenous H-Ras and K-Ras

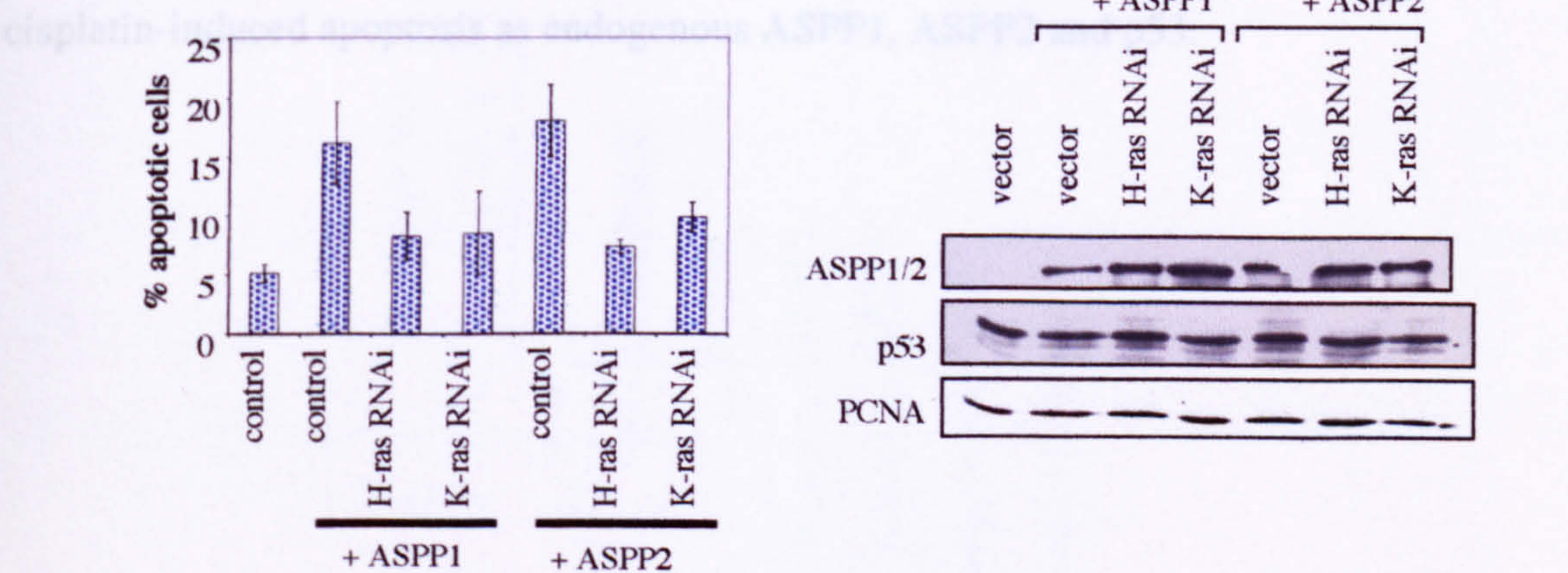


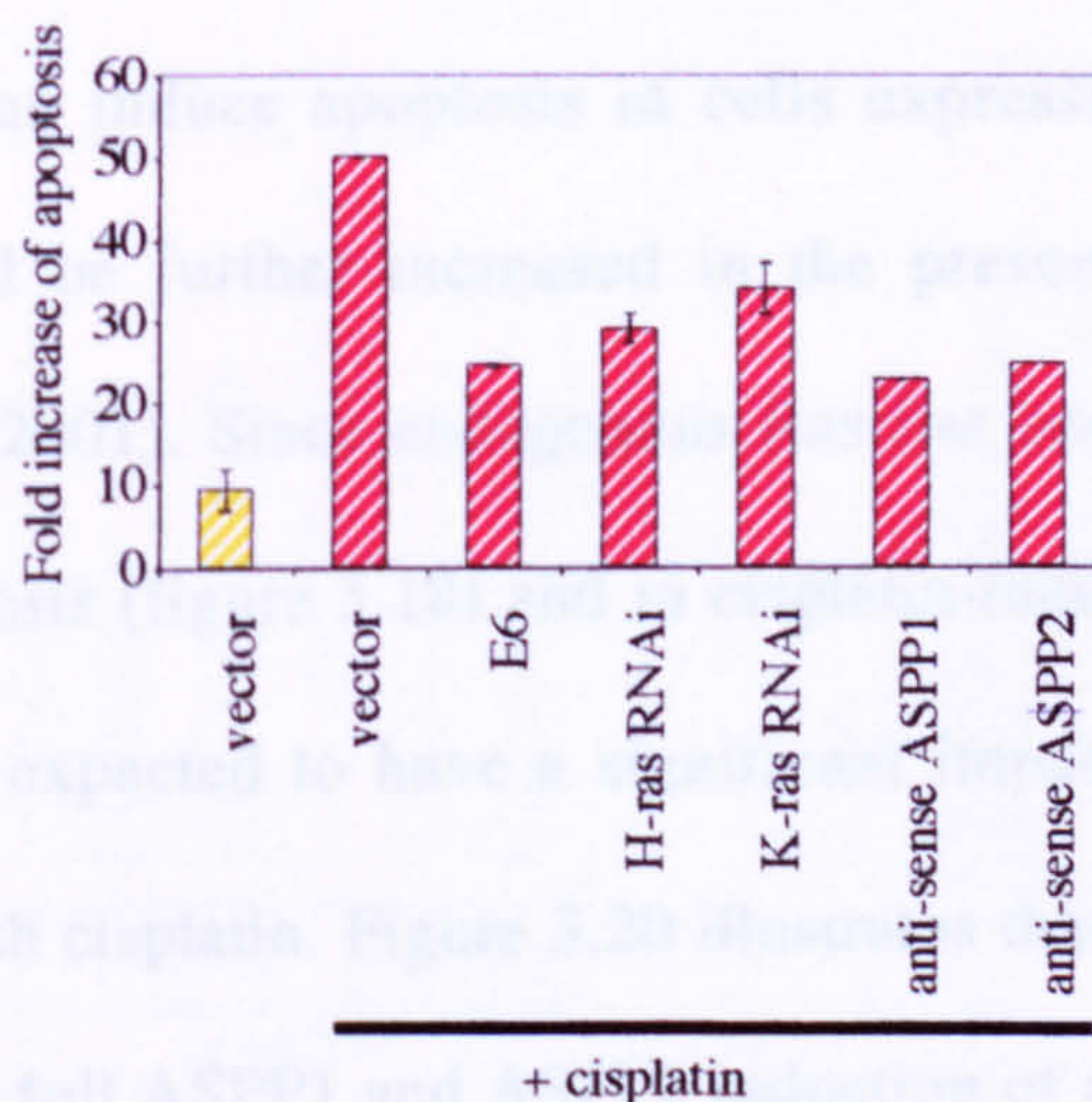
Figure 3.18 Endogenous H-ras and K-ras can induce ASPP1- and ASPP2-mediated apoptosis. FACS analysis of (A) U2OS and (C) MCF7 cells transfected with 10 μ g ASPP1 or ASPP2 in the presence of 9 μ g H-ras RNAi or K-ras RNAi. (B) and (D) Duplicates of each sample from U2OS and MCF7 cells, respectively, were used for western blotting. ASPP1 and ASPP2 were detected with the LX054.1 antibody that cross-reacts with both proteins, and p53 and PCNA were detected with DO-1 and PC-10, respectively. These results are the averages of two independent experiments. This experiment was done in collaboration with Daniele Bergamaschi.

3.2.13.4. Cisplatin-induced apoptosis requires endogenous H-Ras and K-Ras

Cisplatin is a known DNA damaging agent and can induce apoptosis via the p53 pathway (Fritsche et al., 1993; Fujiwara et al., 1994). It is known that ASPP1 and ASPP2 are involved in cisplatin-induced apoptosis (Samuels-Lev et al., 2001). Since endogenous Ras was known to be required for exogenous ASPP1 and ASPP2 apoptotic activity, I tested whether endogenous Ras was involved in cisplatin-induced apoptosis. To address this issue U2OS and MCF7 cells were transfected with Ras RNAi and treated with cisplatin (figure 3.19). Both H-Ras RNAi and K-Ras RNAi reduced cisplatin-induced apoptosis by half. As a control the p53 inhibitor E6, anti-sense ASPP1 and anti-sense ASPP2 were transfected before cisplatin treatment and they too reduced the percentage of apoptotic cells. Endogenous Ras therefore seems to be as necessary to cisplatin-induced apoptosis as endogenous ASPP1, ASPP2 and p53.

3.2.12.3 Endogenous Ras is involved in ASPP1- and ASPP2-mediated apoptosis in cells treated with cisplatin

A.



B.

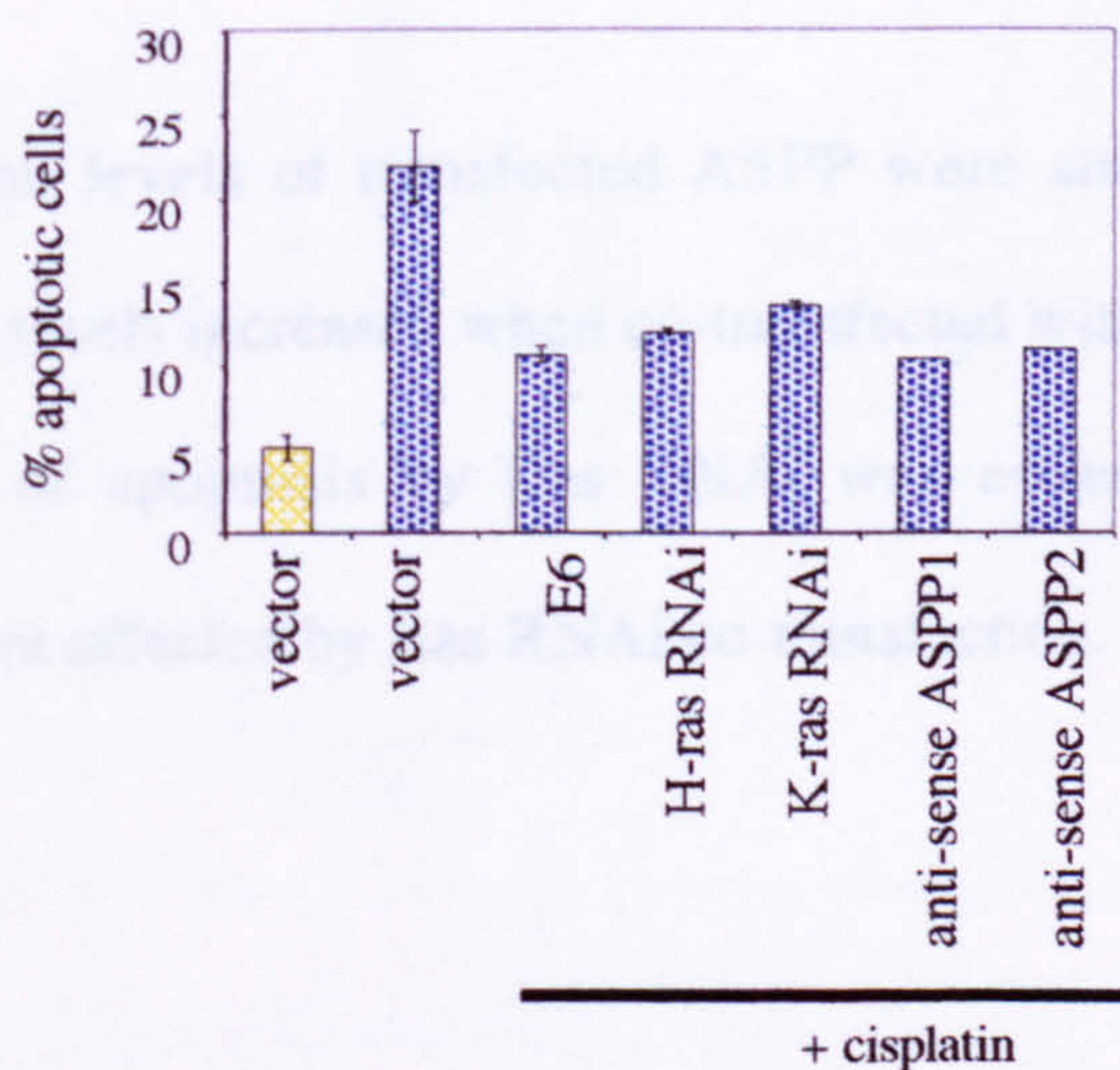


Figure 3.19 Endogenous *H-ras* and *K-ras* are necessary for cisplatin-induced apoptosis. (A) U2OS and (B) MCF7 cells were transfected with 6 μ g E6, 10 μ g *H-ras* RNAi or *K-ras* RNAi, or 15 μ g anti-sense ASPP1 or anti-sense ASPP2 and subsequently treated with 3.5 μ g/ μ l cisplatin for 24 hours. Cells were collected for FACS analysis and the percentage of cells in sub-G1 shown above. This experiment was done in collaboration with Daniele Bergamaschi.

3.2.13.5. Endogenous Ras is involved in ASPP1- and ASPP2-mediated apoptosis in cells treated with cisplatin

Although cisplatin can induce apoptosis in cells expressing p53, the percentage of apoptotic cells could be further increased in the presence of ASPP1 and ASPP2 (Samuels-Lev et al., 2001). Since endogenous Ras has been shown to be involved in ASPP-induced apoptosis (figure 3.18) and in cisplatin-induced apoptosis (figure 3.19) Ras RNAi might be expected to have a significant impact on cells transfected with ASPP and treated with cisplatin. Figure 3.20 illustrates that endogenous H-Ras and K-Ras are necessary for full ASPP1 and ASPP2 induction of apoptosis in both U2OS and MCF7 cells treated with cisplatin. Removal of endogenous Ras by RNAi resulted in a significant reduction of apoptotic cells after ASPP transfection and cisplatin induction.

When the expression levels of transfected ASPP were analyzed by Western blot it seemed that ASPP1 levels increased when co-transfected with Ras RNAi. This suggests that the inhibition of apoptosis by Ras RNAi was underestimated. ASPP2 levels however did not seem affected by Ras RNAi co-transfection.

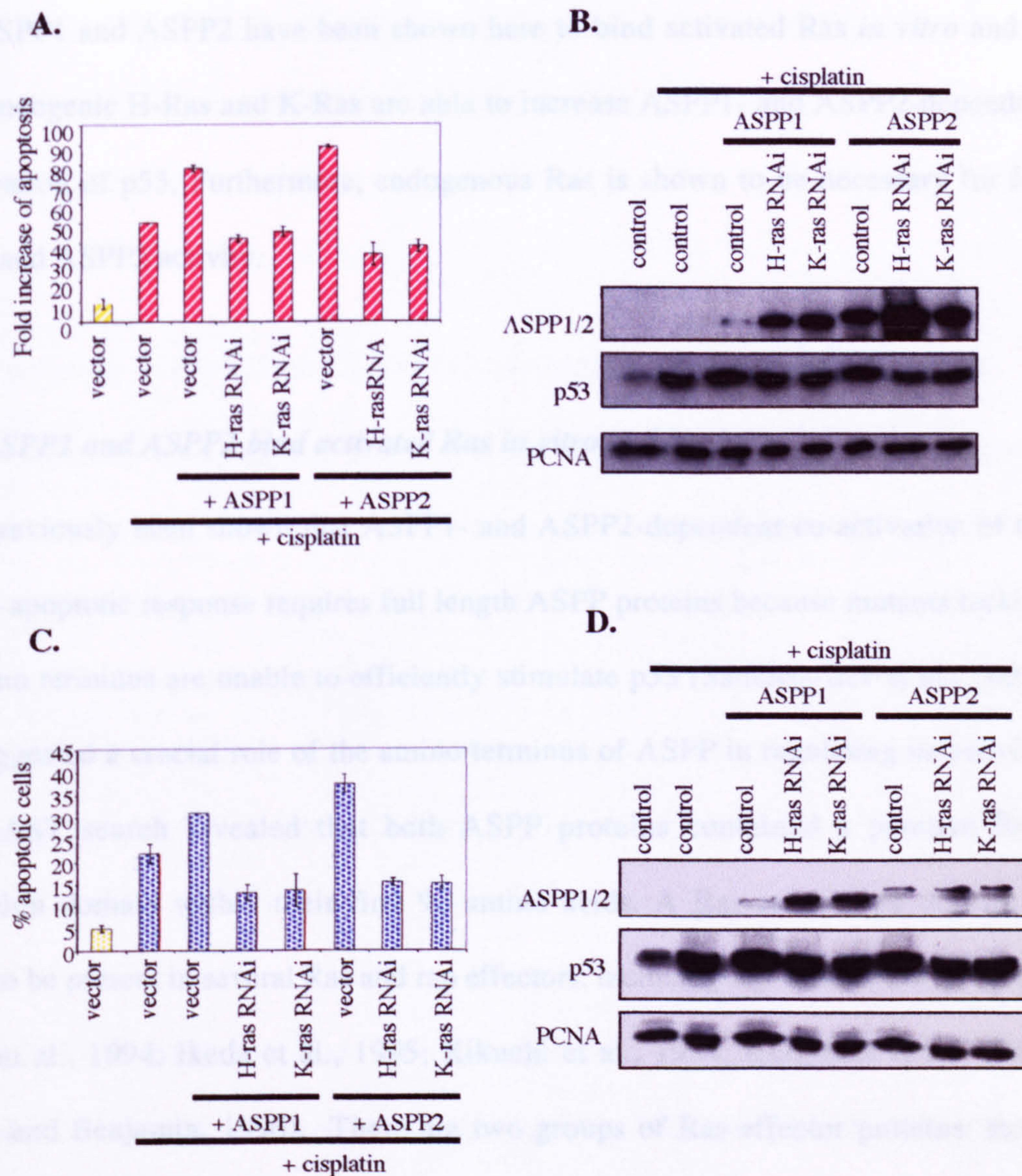


Figure 3.20 Endogenous *ras* is involved in ASPP1- and ASPP2-mediated apoptosis in cells treated with cisplatin. (A) U2OS and (C) MCF7 cells were transfected with 10 μ g ASPP1 or ASPP1, in the presence of 9 μ g H-ras RNAi or K-ras RNAi. The cells were subsequently treated with 3.5 μ g/ μ l cisplatin for 24 hours and collected for FACS analysis. Duplicates of the samples were used to detect ASPP1, ASPP2, p53 and PCNA levels by Western blot in (B) U2OS and (D) MCF7 cells. This experiment was done in collaboration with Daniele Bergamaschi.

3.3 Discussion

Both ASPP1 and ASPP2 have been shown here to bind activated Ras *in vitro* and in cells. Oncogenic H-Ras and K-Ras are able to increase ASPP1- and ASPP2-dependent co-activation of p53. Furthermore, endogenous Ras is shown to be necessary for full ASPP1 and ASPP2 activity.

3.3.1. ASPP1 and ASPP2 bind activated Ras *in vitro* and *in vivo*

It has previously been shown that ASPP1- and ASPP2-dependent co-activation of the p53 pro-apoptotic response requires full length ASPP proteins because mutants lacking the amino terminus are unable to efficiently stimulate p53 (Samuels-Lev et al., 2001). This suggested a crucial role of the amino terminus of ASPP in regulating its activity. My BLAST search revealed that both ASPP proteins contained a putative Ras-association domain within their first 90 amino acids. A Ras-association domain is known to be present in several Ras and rap effectors, including AF-6, RalGDS and RGL (Hofer et al., 1994; Ikeda et al., 1995; Kikuchi et al., 1994; Kuriyama et al., 1996; Ponting and Benjamin, 1996). There are two groups of Ras effector proteins: those containing the Ras-binding domain, such as raf, and those containing the Ras-association domain. Although the sequence of the Ras-association domain is not homologous to the Ras-binding domain, the majority of hydrophobic residues conserved among Ras-association sequences are also conserved in Ras-binding sequences (Ponting and Benjamin, 1996).

I have shown here that ASPP1 and ASPP2 bind preferentially to activated Ras, both *in vitro* and *in vivo*. The ability to selectively bind active Ras is common for Ras effectors such as Raf, RalGDS and PI3K (Hofer et al., 1994; Ikeda et al., 1995; Kikuchi et al.,

1994; Rodriguez-Viciano et al., 1994; Van Aelst et al., 1993; Vojtek et al., 1993). The fact that Ras can bind directly to ASPP1 and does so in a GDT/GTP dependent manner both *in vitro* and *in vivo* is a strong indication that the ASPP proteins might be novel effectors of Ras.

3.3.2. Oncogenic H-RasV12 and K-RasV12 stimulate ASPP1 and ASPP2 activity

Once bound to its effectors, Ras activates them and leads to a downstream signaling cascade. Since ASPP1 and ASPP2 are putative effectors of Ras, the effect of Ras on their activity was analyzed. Ras stimulates its effectors when in its GTP-bound form. As such, a constitutively active mutant was used to analyze the effect of Ras on ASPP activity. The most common mutation of Ras in tumours occurs in its twelve codon, resulting in a change of amino acid from glycine to valine. The RasV12 mutant is constitutively active due to a reduced GTPase activity (Sweet et al., 1984).

H-RasV12 and K-RasV12 mutants were therefore tested for their effect on ASPP1 and ASPP2 activity using transient transactivation assays. Both oncogenic Ras genes were found to stimulate ASPP activity approximately 2.5 fold.

3.3.3. Oncogenic Ras increases ASPP2 stimulation of p53 family members

ASPP1 and ASPP2 are able to increase the pro-apoptotic activity of other p53 family members, namely p63 and p73 (Bergamaschi et al., 2004). To investigate whether oncogenic Ras stimulates ASPP2 synergy with all p53 family members, oncogenic Ras was co-transfected with ASPP2 and either p53, p63 or p73. Oncogenic Ras was able to increase ASPP2 co-activation of p63 and p73 to the same extent as p53, namely 2.5

fold. The fact that oncogenic Ras can equally increase ASPP2 stimulation of each p53 family member supports the suggestion that Ras does not stimulate p53 directly but does so via the ASPP protein.

Ras is known to induce p53 activity via the stimulation of the Arf protein which inhibits Mdm2-mediated degradation of p53 (see section 1.4.5) (Pantoja and Serrano, 1999; Serrano et al., 1997; Weber et al., 1999). Neither p63 nor p73 can be degraded by Mdm2 (Balint et al., 1999; Little and Jochemsen, 2001; Lohrum and Vousden, 1999). Therefore, the co-activation of these pro-apoptotic proteins by Ras is likely to be independent of Arf. This confirms ASPP as an intermediate in a new signaling pathway between Ras and p53.

3.3.4. Endogenous Ras is necessary for full ASPP1 and ASPP2 activity

A dominant negative form of H-Ras known as H-rasN17, inhibits ASPP2 and p53 synergy in a transient transactivation assay, suggesting that endogenous Ras is inhibited by the dominant negative Ras and raising the question of whether endogenous Ras has any effect on ASPP1 and ASPP2 activity. To address this issue, H-Ras RNAi and K-Ras RNAi were constructed.

The RNAi constructs used in this chapter were made against endogenous Ras. However, since only oncogenic Ras was available as a tagged version, oncogenic Ras was used to test the efficacy of the RNAi constructs. A tagged protein was necessary to allow detection of transfected Ras as levels of endogenous Ras are high. The H-Ras RNAi was not expected to differentiate between the oncogenic and wild-type version of H-Ras since the nucleotides selected for the construct represented the sequence 299-315

downstream of the start site whereas the single point mutation occurs at position 35. On the other hand, the K-Ras RNAi sequence, which represented nucleotides 25-43, overlapped with the point mutation situated at nucleotide 34. RNAi has been shown to be very specific with a single nucleotide difference capable of preventing its repression of the gene of interest (Brummelkamp et al., 2002). However the K-RNAi construct in pSUPER shown in this chapter was nonetheless capable of reducing K-RasV12 expression specifically, compared to H-RasV12. We could expect that this K-Ras RNAi is capable of reducing wild-type RNAi in a more efficient manner, suggesting an underestimation of the repression by K-Ras RNAi in our system.

Unfortunately, my transfection efficiency was too low to detect the effect of my RNAi constructs on endogenous Ras. In order to test the efficacy of the K-Ras RNAi and H-Ras RNAi on endogenous K-Ras and H-Ras respectively, these constructs could be transfected into cells with wild type endogenous Ras and the transfected cells selected. This can be done, for example, by magnetic selection of the transfected cells using a system such as MACS. An alternative method to see the effect of RNAi on endogenous Ras levels would be to infect cells using a retroviral system containing our RNAi construct. It would clearly be important to verify these conclusions in cell lines stably expressing Ras RNAi. Alternatively, the RNAi efficiency could be tested by analyzing H-Ras and K-Ras mRNA levels; if the protein has a long half life, the effect of the RNAi might be seen more clearly at the mRNA rather than the protein level. Regrettably, due to time constraints, I have been unable to try these experiments. However, the data in this chapter shows that H-Ras RNAi and K-Ras RNAi both have an effect in our system, strongly suggesting that they have an effect on endogenous Ras expression levels.

Transactivation assays and FACS analysis showed that inhibition of the expression of endogenous H-Ras and K-Ras by RNAi resulted in a significant decrease in ASPP1 and ASPP2 co-activation activity. This inhibition occurred both in the presence and absence of the DNA damaging drug cisplatin showing a propensity of Ras to inhibit ASPP in both a high and low apoptotic background. Cisplatin-induced apoptosis was decreased by Ras RNAi in the absence of exogenous ASPP. The possibility that this inhibition was via endogenous ASPP was supported by the decrease in cisplatin-induced apoptosis in the presence of ASPP1 and ASPP2 anti-sense nucleotides. Unfortunately, we have been unable to construct an efficient RNAi against ASPP1 or ASPP2 in our laboratory; however, the anti-sense nucleotides used in these experiments have previously been shown to inhibit ASPP1 and ASPP2 expression (Samuels-Lev et al., 2001).

In the FACS analysis experiments looking at the effect of Ras RNAi on ASPP1 and ASPP2 apoptotic activity, Western blots were performed to detect ASPP1, ASPP2 and p53 expression levels. Ras RNAi did not significantly affect ASPP1, ASPP2 and p53 levels when co-transfected in the absence of cisplatin treatment. However, in cells which were transfected with ASPP and Ras RNAi and subsequently treated with cisplatin, the levels of transfected ASPP1 increased significantly in the presence of both H-Ras RNAi and K-Ras RNAi. This suggests that the inhibition measured of ASPP1 activity by Ras RNAi was underestimated since the protein levels of ASPP1 were higher in the presence of Ras RNAi. ASPP2 and p53 levels, however were not significantly affected by the presence of Ras RNAi.

3.3.5. Cell staining shows a difference in ASPP1 and ASPP2 response to oncogenic Ras

Both ASPP1 and ASPP2 can bind activated Ras and both proteins are equally stimulated by oncogenic Ras and endogenous wild type Ras activity. It was therefore expected that ASPP1 and ASPP2 would have the same pattern of cell staining in the presence of oncogenic Ras.

Interestingly, when ASPP1 was co-transfected with oncogenic Ras, its sub-cellular localization changed dramatically. The staining was much brighter than in cells not co-transfected with Ras and, when co-transfected with Ras, ASPP1 formed doughnut-type shapes in the cytoplasm. These unusual shapes did not localize with Ras and there seemed to be no co-localization between the two proteins.

The lack of co-localization is very surprising as ASPP1 has been shown to bind *in vitro* and *in vivo* to Ras and the *in vivo* binding has shown that ASPP1 has an even greater affinity to Ras than does ASPP2. One possibility for not seeing co-localization between ASPP1 and Ras might be due to the antibody used against ASPP1 in the cell staining. ASPP1 is tagged with a V5 epitope in the carboxy-terminus and the V5 antibody was used in the immunofluorescence assay. There is a possibility that ASPP1 might exist as splice variants and some splice forms might lack the amino terminus region. Using the V5 epitope, only the ASPP1 fragments with the carboxy-terminus present are recognized. Since the Ras-association domain of ASPP1 is in its amino terminus only the ASPP1 fragments containing this domain would associate with Ras. Therefore the immunofluorescence should be repeated with an antibody against ASPP1 that has an epitope in the amino terminus part of the protein. The fact that ASPP2 is seen to co-

localize with Ras using the V5 antibody against the carboxy-terminus tag of ASPP2 makes the above explanation unlikely, however, unless there is a difference in splice formation of ASPP1 and ASPP2. Very recent current work in our laboratory has revealed by Western Blotting that ASPP1 has additional bands in some circumstances, although only one band for ASPP2 has only ever been seen. These results are preliminary and much work needs to be done before the presence of ASPP1 splice variants can be confirmed.

The difference in ASPP1 co-localization after oncogenic Ras co-transfection remains a mystery. The increase in ASPP1 brightness correlates with the amount of oncogenic Ras co-transfected: the higher the levels of Ras transfected in the cell, the brighter the pattern of the ASPP1 doughnut-like shapes.

There is little known about the difference in ASPP1 and ASPP2 function. Up to now both proteins seem to have the same effectors, namely the p53-family members, and they are both stimulated to the same extent by Ras. The expression levels in various tissues are also similar (unpublished data). However, it is clear that there is a functional difference between the two proteins as ASPP2 knock-out mice have a very strong phenotype (Vives et al., under review). It has also been shown that ASPP1 and ASPP2 have different binding affinities to a mutant form of p53: ASPP1 cannot bind to p53(181L) or p53(181C) mutant p53 whereas ASPP2 does not differentiate between these mutants and wild type p53 in its binding affinity (Samuels-Lev et al., 2001). Although the difference in ASPP1 and ASPP2 cell staining pattern in the presence of oncogenic Ras does not help to explain how these proteins differ, it does confirm that there is a distinction in the functions of ASPP1 and ASPP2. The fact that higher

organisms carry both ASPP1 and ASPP2 genes suggests that there must be an evolutionary reason to select for both genes instead of only one of them. Further research will undoubtedly yield a functional difference between these two family members.

3.4. Scope of study

ASPP1 and ASPP2 are two new potential Ras effectors, binding Ras both *in vitro* and *in vivo* with a higher affinity for active Ras-GTP than for Ras-GDP. The ASPP proteins do so via their Ras-association domain which is present in their first 90 amino acids. As with many Ras effectors, Ras is capable of stimulating ASPP activity, shown here with transactivation assays. Both H-RasV12 and K-RasV12 oncogenic mutants can increase ASPP1 and ASPP2 stimulation of p53 pro-apoptotic activity 2.5 fold. Oncogenic H-RasV12 can also increase ASPP2 stimulation of the p53 family members p63 and p73. Endogenous H-Ras and K-Ras have been shown to be necessary for full ASPP1 and ASPP2 pro-apoptotic activities using Ras RNAi, in both transactivation and FACS assays.

I have shown that ASPP1 and ASPP2 can link the Ras and p53 signalling pathways. Unlike a pathway previously described where Ras has been shown to induce p53-dependent senescence via Arf (Pantoja and Serrano, 1999; Serrano et al., 1997; Weber et al., 1999), the pathway described in this chapter shows that Ras can stimulate p53-dependent apoptosis via the activation of ASPP1 and ASPP2.

Chapter 4

Ras stimulates ASPP1 and ASPP2 via the Raf-MEK-MAPK pathway

4.1 Introduction

Ras acts as a point of convergence linking various extracellular signals to a number of different signalling pathways. It responds to growth factors, cytokines, hormones and neurotransmitters via stimulation of cell surface receptors. Following the necessary stimulus, Ras becomes activated by guanine nucleotide exchange factors (GNEFs) which can replace the GDP nucleotide with a GTP nucleotide, resulting in an active form of Ras (Ma and Karplus, 1997; Wittinghofer and Pai, 1991). Once GTP-bound, Ras changes its tertiary structure with two regions displaced: the switch I and the switch II regions. These are the regions that encompass the binding site to the effector proteins. Therefore, the interaction of Ras with its downstream effectors is only possible after the exchange of GDP for GTP.

Biochemical analysis, genetic screening and yeast two hybrid screening in a variety of systems ranging from *C.elegans*, *S. cerevisiae*, *Drosophila* and human cells have led to the discovery of a number of downstream Ras effector pathways. The three most studied downstream effector pathways are the Raf-MAPK pathway, the phosphoinositol 3-kinase (PI3K) pathway and the RalGDS, although other putative downstream Ras pathways are currently under investigation, such as the p120-GAP, AF-6, Nore1 and PKC ζ .

Ras-GTP has been shown to bind PI3K and activate its p110 catalytic domain (Rodriguez-Viciano et al., 1996; Rodriguez-Viciano et al., 1994). PI3K acts as a lipid

kinase that phosphorylates phosphoinositides at the 3' position of the inositol ring, resulting in a phosphatidyl inositol (3,4,5)-triphosphate (PtdIns[3,4,5]P₃) lipid. PI3K activity can lead to Akt/PKB stimulation which is believed to be mediated by PKB binding to the PtdIns[3,4,5]P₃ via its PH domains (Franke et al., 1997a; Franke et al., 1997b; Klippel et al., 1997). Much interest has been shown in PKB function as studies have indicated that it plays a crucial role in Ras-mediated survival (Addison et al., 1990; D'Mello et al., 1997; Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Khwaja et al., 1997; Kulik et al., 1997; Yao and Cooper, 1995).

The presence of RalGDS as a Ras effector of Ras suggests cross-talk between the various Ras family proteins. RalGDS is a guanine nucleotide exchange factor for the two Ras-related proteins RalA and RalB. Ras-GTP can therefore lead to stimulation of RalA and RalB via the RalGDS effector pathway (Urano et al., 1996; Wolthuis et al., 1998).

The most studied downstream signalling pathway of Ras is the Raf-MEK-MAPK phosphorylation cascade. Active Ras-GTP but not Ras-GDP can bind to Raf directly (Van Aelst et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993) and recruit it to the plasma membrane (Leevers et al., 1994; Stokoe et al., 1994). Raf-1 contains a Ras-binding domain (RBD) within its residues 51-131 through which it interacts with Ras (Clark et al., 1996; Nassar et al., 1995; Vojtek et al., 1993). Once Ras and Raf-1 have associated, a second RBD present in Raf-1 contacts Ras-GTP. This second contact site is within the cysteine-rich domain (CRD) of Raf-1 (Brtnva et al., 1995; Drugan et al., 1996; Mott et al., 1996). Ras alone is not, however, sufficient to fully activate Raf. The phospho-serine binding protein 14-3-3 is thought to regulate

Raf-1 catalytic activity (Fantl et al., 1994; Freed et al., 1994) and the presence of the anionic membrane phospholipid, phosphatidylserine, can bind and activate Raf-1 via its CRD domain (Ghosh et al., 1994). Mutational analysis has shown that tyrosines phosphorylated at residues 340 and 341 leads to enhanced Raf-1 catalytic activity (Marais et al., 1995).

Once active, Raf can phosphorylate and activate MAPK extracellular signal-regulated kinase, also known as MEK (Ahn et al., 1991; Cowley et al., 1994; Dent et al., 1992; Gomez and Cohen, 1991; Huang et al., 1993; Kosako et al., 1992; Kyriakis et al., 1992; Nakielny et al., 1992). In turn, MEK can then phosphorylate and activate the two mitogen-activating protein kinases, MAPK1 and MAPK2, also known as Erk1 and Erk2 (Crews et al., 1992; Kosako et al., 1992; Matsuda et al., 1992). Activated MAPKs homodimerize and translocate to the nucleus (Khokhlatchev et al., 1998), where they can phosphorylate and stimulate a range of substrates such as p90 S6K and transcription factors, including Elk-1 and Ets-2 (Chen et al., 1992; Marais et al., 1993; Marshall, 1995). These transcription factors regulate the expression of immediate-early genes such as *c-fos*, eventually leading to proliferation.

Although downstream signalling pathways of Ras are often involved in growth, differentiation or survival, Ras is also known to regulate apoptosis. MEKK1 can bind Ras in a GTP-dependent manner and is activated by Ras under stress conditions. Once active, MEKK1 can activate its downstream effector JNKK, also known as SAPKK, which in turn activates and phosphorylates JNK/SAPK (Derijard et al., 1994; Minden et al., 1994; Yan et al., 1994). In turn JNK/SAPK can activate a number of transcription factors such as c-Jun and ATF-2. Both these transcription factors are involved in

inducing apoptosis (Chen et al., 1996b; Verheij et al., 1996; Xia et al., 1995). Although the Raf-MAPK pathway is usually seen to regulate growth and proliferation, the reality has been shown to be more complex with the discovery that the same pathway can also induce apoptosis (Kauffmann-Zeh et al., 1997). Yet another Ras effector, Nore1, has also been shown to induce apoptosis in response to Ras (Khokhlatchev et al., 2002)

Ras is therefore upstream of many different pathways and its activation can result in a wide range of responses such as growth, proliferation, differentiation, senescence and apoptosis. The response to Ras activation is thought to depend on various factors such as the duration of extracellular factor stimulation and cell type. Much is still unknown about which response Ras will stimulate once activated.

I have shown in the previous chapter that ASPP1 and ASPP2 activity are stimulated by Ras. In this chapter the signalling pathway leading to ASPP stimulation by Ras is investigated. ASPP1 and ASPP2 are shown to be stimulated by the Raf-MEK-MAPK pathway and to be phosphorylated both *in vitro* and *in vivo* by MAPK, resulting in increased ASPP protein stability.

4.2 Results

4.2.1. Ras activates ASPP2 via the Raf pathway

The most studied downstream signaling pathway of Ras is the Raf-MAPK pathway. To ascertain whether Ras can activate ASPP directly or whether it does so via the Raf pathway, an activated form of Raf known as Raf CX was used. The Raf CX form contains an artificial CAAX motif in its carboxy terminus which signals for isoprenylation, resulting in the protein being localized to the cell membrane therefore rendering it active.

Co-transfection of ASPP2 and p53 with Raf CX in Saos2 cells leads to an increase in Bax-luciferase activity (figure 4.1A). This increase in p53 transactivation activity is probably via the ASPP2 protein as p53 co-transfected with RafCX alone does not have any increase in activity. RafCX can increase ASPP2 and p53 activity to the same extent as oncogenic H-RasV12 and K-RasV12 do, namely 2-2.5 fold, suggesting that oncogenic Ras activates ASPP via the Raf pathway (figure 4.1D).

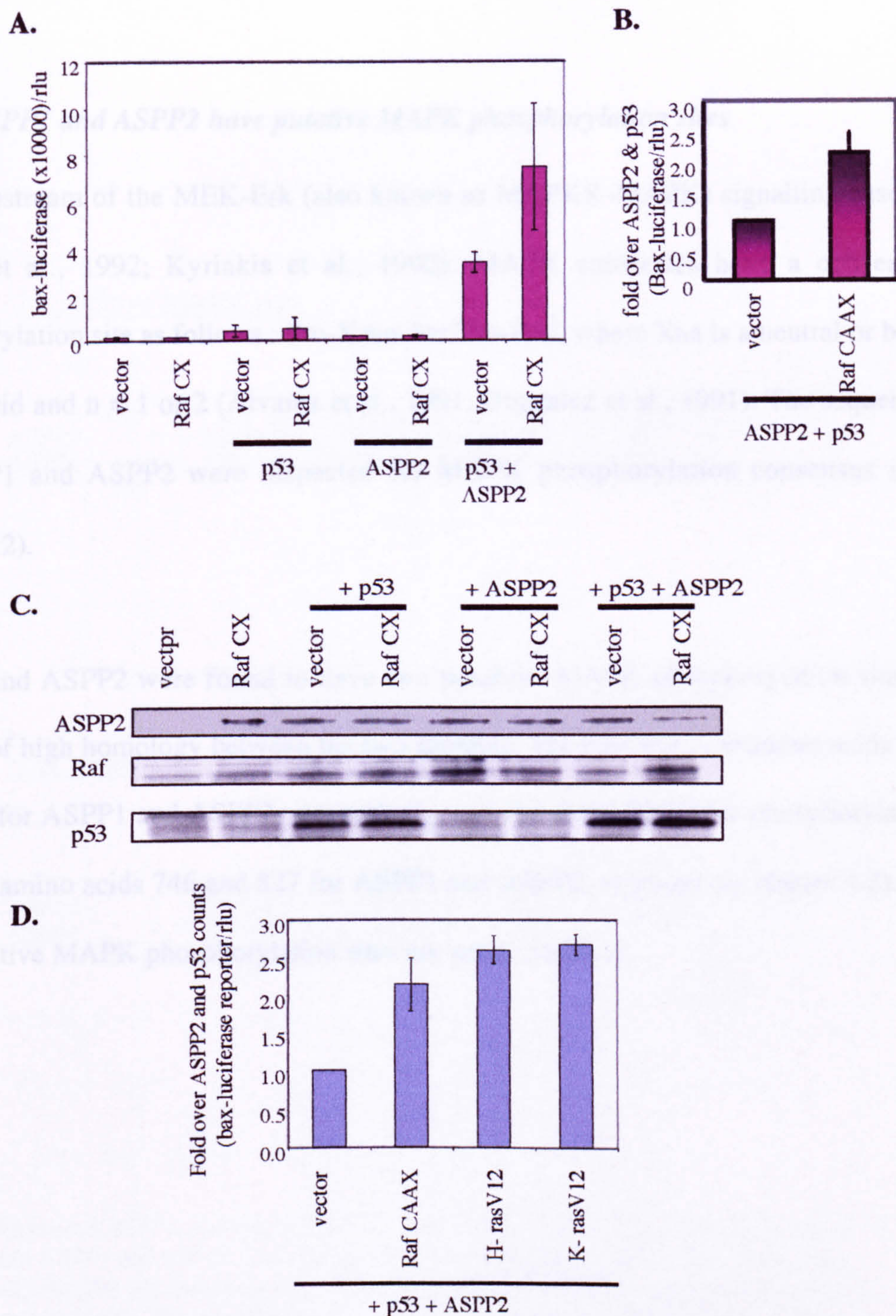


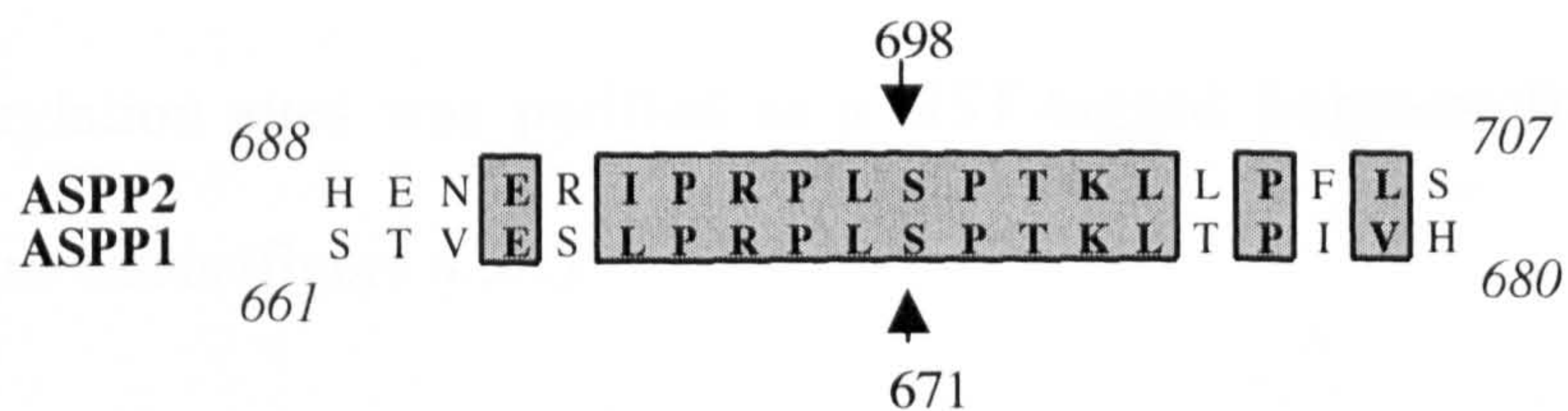
Figure 4.1 Activated Raf increases ASPP2 activity to a similar extent as oncogenic ras. (A) Saos2 cells were transfected with 4 μ g ASPP2, 50 ng p53 and 1.5 μ g RafCX expression plasmids as indicated. All samples were co-transfected with Bax-luciferase. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. (B) The Bax-luciferase value of p53 and ASPP2 co-transfected was arbitrarily assigned as a value of 1.0 to allow estimation of the effect of RafCX. (C) Part of the lysates from A was subjected to western blotting and ASPP2 was detected with the 5410 antibody, Raf with the C-12 antibody and p53 with the DO-1 antibody. (D) Comparing the effect of Raf on ASPP2 and p53 with the effect of oncogenic H-ras and K-ras. The value of p53 and ASPP2 co-transfected with Bax-luciferase is taken as a value of 1.0 to allow estimation of the effects of RafCX, H-rasV12 and K-rasV12. This is an average of at least three independent experiments.

4.2.2. ASPP1 and ASPP2 have putative MAPK phosphorylation sites

Raf is upstream of the MEK-Erk (also known as MAPKK-MAPK) signalling cascade (Howe et al., 1992; Kyriakis et al., 1992). MAPK substrates have a consensus phosphorylation site as follows : Pro-Xaa-Ser/Thr-Pro , where Xaa is a neutral or basic amino acid and n = 1 or 2 (Alvarez et al., 1991; Gonzalez et al., 1991). The sequences of ASPP1 and ASPP2 were inspected for MAPK phosphorylation consensus sites (figure 4.2).

ASPP1 and ASPP2 were found to have two putative MAPK phosphorylation sites at regions of high homology between the two proteins. The first site is at amino acids 671 and 698 for ASPP1 and ASPP2, respectively, and the second putative phosphorylation site is at amino acids 746 and 827 for ASPP1 and ASPP2, respectively (figure 4.2). All four putative MAPK phosphorylation sites are serine residues.

1st putative MAPK site:



2nd putative MAPK site:

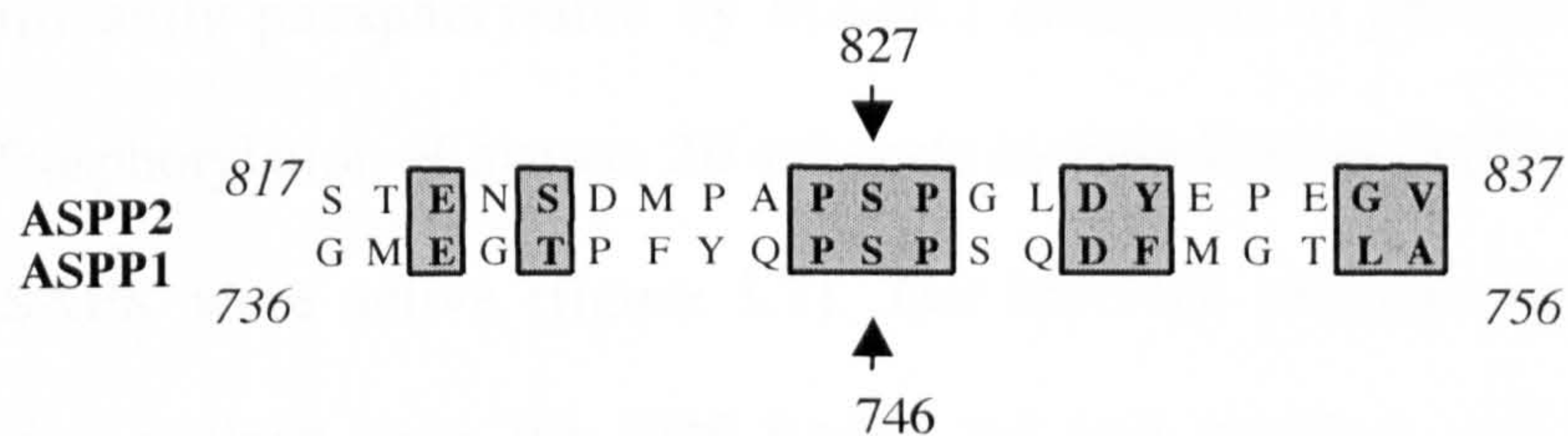


Figure 4.2 *ASPP1* and *ASPP2* contain two putative MAPK phosphorylation sites. Both *ASPP1* and *ASPP2* have two putative phosphorylation sites in their C-terminus and both sites are situated in regions of homology between the two proteins. The first putative MAPK phosphorylation site of *ASPP1* is at amino acid 671 and the second is at amino acid 746. The first putative phosphorylation site of *ASPP2* is at amino acid 698 and the second one at amino acid 827. All four putative MAPK phosphorylation sites are serines.

4.2.3. ASPP2 is phosphorylated *in vitro* by MAPK

To perform *in vitro* phosphorylation assays on ASPP2, purified recombinant ASPP2 was needed. Since full length ASPP2 was too large to make a recombinant protein, the carboxy terminus of ASPP2 (amino acids 693-1128) containing both putative MAPK phosphorylation sites was purified as a GST-tagged fragment from *E. coli* using glutathione beads (figure 4.3A).

The recombinant ASPP2 fragment was then mixed with recombinant MAPK1 or p38 SAPK (provided by Dario Alessi's laboratory at the university of Dundee) in the presence of ^{32}P -labelled ATP. Figure 4.3C shows that the ASPP2 fragment (693-1128) was significantly phosphorylated by MAPK1 compared to phosphorylation by p38 SAPK. Phosphorylation of histone 2B substrate by both kinases confirmed that MAPK1 and p38SAPK were active (figure 5.7). The MAPK1 phosphorylated fragment of ASPP2 was excised from the SDS-PAGE gel and purified, and its radioactivity measured (figure 4.3D). The fragment was subsequently digested with trypsin and then fractionated on a high performance liquid chromatography (HPLC) C-18 column. An acetonitrile gradient was used to elute the trypsinized fragments: the smaller, hydrophilic fragments were eluted first and the larger hydrophobic fragments were eluted as the acetonitrile gradient increased. All phosphorylated fractions were expected to be ^{32}P labelled. To identify the phosphorylated fractions, the ^{32}P content of each fraction was measured as they were eluted from the column and the cpm noted. A graph showing the cpm counts of the fractions showed two radioactive peaks (figure 4.3E). The fractions representing these radioactive peaks were analyzed by the mass spectrometer service at the Protein Phosphorylation Unit, University of Dundee, to measure their molecular mass.

Using a programme that could predict the mass and sequences of the ASPP2 fragments after digestion with trypsin, the phosphorylated fractions were matched to the appropriate sequences. The first radioactive peak eluted from the HPLC column was found to be the linker region between the GST tag and the recombinant ASPP2 fragment. The second radioactive peak corresponded to a fragment of the same mass as the fragment containing the second putative phosphorylation site, namely serine 827. However, because of the unusually large size of the radioactive peptide fraction due to the lack of trypsin recognition sites, Edman degradation was not performed so there was no confirmation that it was the serine 827 that was phosphorylated. Nonetheless, computer analysis showed no other putative MAPK phosphorylation sites in the large fraction representing the second radioactive peak and it was therefore assumed that it was serine 827 that was phosphorylated *in vitro* by MAPK.

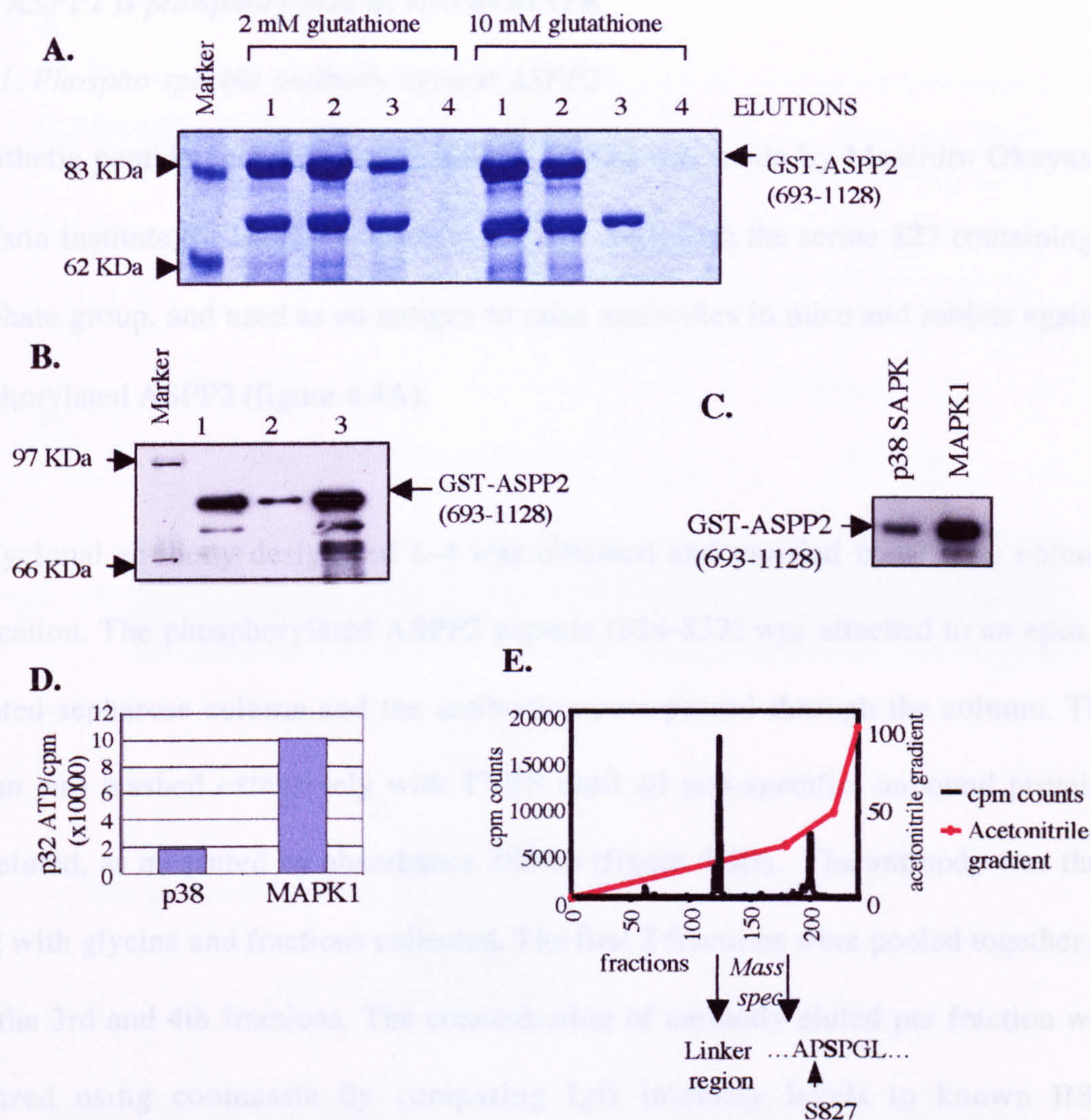


Figure 4.3 *ASPP2 is phosphorylated in vitro by MAPK* (A) The carboxy terminus of ASPP2 (693-1128) was expressed as a GST-fusion protein in BL21 cells and purified with glutathione sepharose beads. The protein was eluted off the beads using glutathione at either 2mM or 10mM and the samples were collected after each of the four washes with glutathione (labelled 1-4). An aliquot was collected, run on an SDS-PAGE gel and the total amount of protein was detected by coomassie staining. Samples 1,2 and 3 from 2mM glutathione and 1 and 2 from 10mM glutathione were pooled and dialyzed in water. The protein was then concentrated and resuspended in 1x kinase buffer. (B) The pooled samples were analyzed by western blotting before (1) and after (3) dialysis and GST-ASPP2 (693-1128) was detected with a 5410 antibody against the carboxy-terminus of ASPP2. An aliquot of elution 3 from 10mM glutathione shown in (A) was run on lane 2. (C) An *in vitro* phosphorylation assay was performed with ^{32}P labelled ATP as described in Materials and Methods with the recombinant ASPP2 fragment in the presence of recombinant p38 SAPK or MAPK. The phosphorylated ASPP2 fragment was resolved on an SDS-PAGE gel, transferred onto nitrocellulose and the ATP levels detected by autoradiography. (D) The intensity of the phosphorylated ASPP2 fragment was measured using the GeneTools from SynGene programme and represented on a bar graph. (E) The MAPK phosphorylated ASPP2 fragment was trypsinized and chromatographed on a Vydac C_{18} column (E, bottom panel). The radioactive peptides were measured by mass spectrometry. The first peak was shown to represent the GST linker region whereas the second represented a region of equal mass to the fragment containing the serine 827.

4.2.4. ASPP2 is phosphorylated in vivo by MAPK

4.2.4.1. Phospho-specific antibody against ASPP2

A synthetic peptide encoding amino acids 824-832 was made by Masahiro Okuyama (Wolfson Institute for Biomedical Research, London), with the serine 827 containing a phosphate group, and used as an antigen to raise antibodies in mice and rabbits against phosphorylated ASPP2 (figure 4.4A).

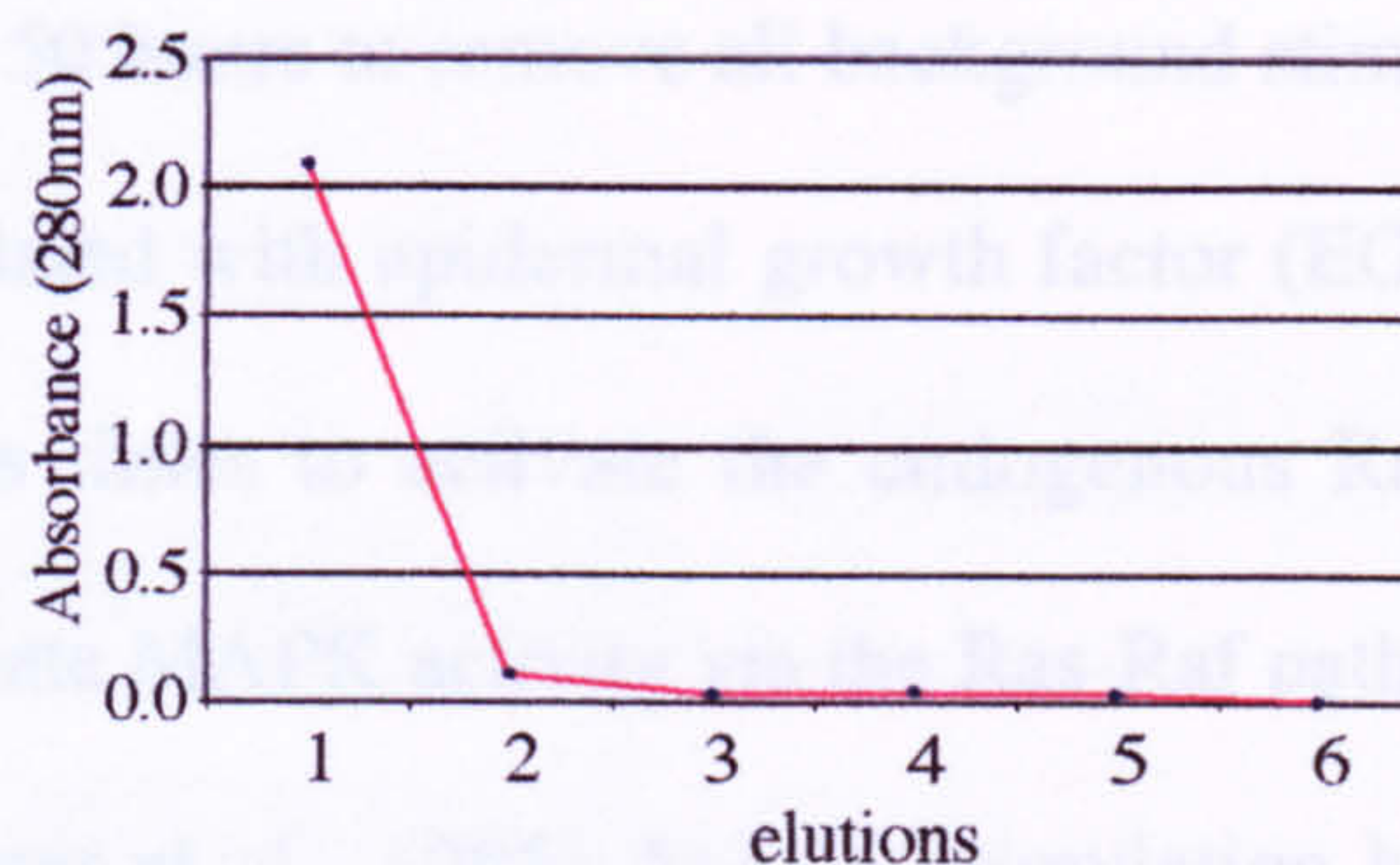
A polyclonal antibody designated S-4 was obtained and purified by affinity column purification. The phosphorylated ASPP2 peptide (824-832) was attached to an epoxy-activated-sepharose column and the antibody serum passed through the column. The column was washed extensively with TTBS until all non-specific, unbound proteins were eluted, as measured by absorbance 280nm (figure 4.4B). The antibody was then eluted with glycine and fractions collected. The first 2 fractions were pooled together as were the 3rd and 4th fractions. The concentration of antibody eluted per fraction was measured using coomassie by comparing IgG intensity levels to known BSA concentrations (figure 4.4C).

To test the efficacy of the purified phospho-specific antibody, a non-radioactive *in vitro* phosphorylation assay was performed on the purified ASPP2 (693-1128) with recombinant MAPK1 in the presence or absence of ATP. The samples were resolved on an SDS-PAGE gel and the phospho-specific ASPP2 antibody was used in Western blot to detect ASPP2 phosphorylated *in vitro*. As shown in figure 4.4D, the phospho-specific antibody recognised phosphorylated ASPP2 specifically.

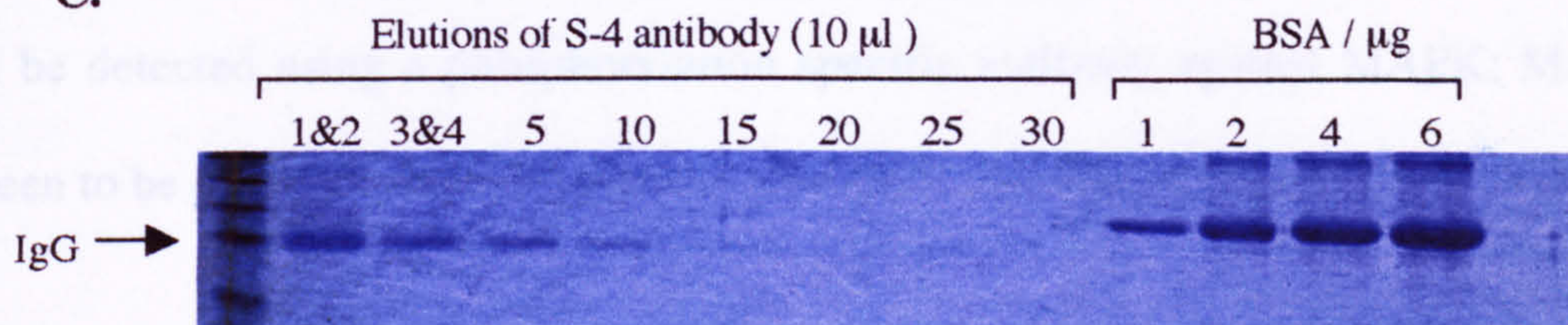
A.

C-PAPS(PO₃H₂)PGLY

B.



C.



D.

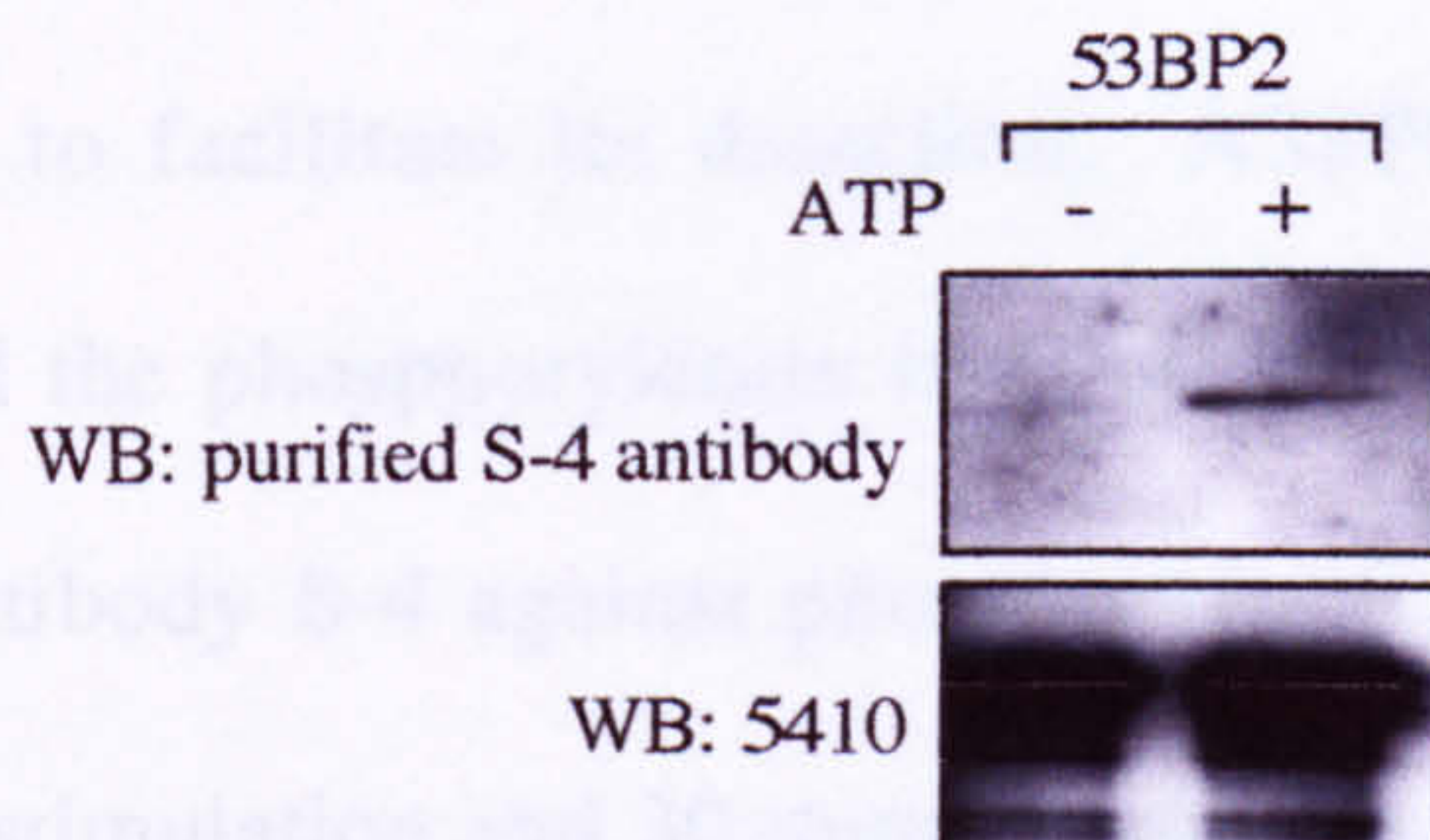


Figure 4.4 *S-4 is a phosphospecific antibody that recognizes ASPP2 phosphorylated at serine 827* (A) A phospho-peptide representing amino acids 824-832 of ASPP2 was synthesized with the serine 827 containing a phosphate group. A cysteine was added to the amino-terminus of the peptide. This peptide was used to raise antibodies in mice and rabbit. (B) A polyclonal antibody labelled S-4 raised against the synthetic phosphopeptide was affinity purified with the phospho-peptide column. After adding the serum to the affinity resin column, the column was washed extensively with TTBS. The flow-through of these washes was collected and the protein concentration measured by absorbance at 280nm. After 6 washes all non-specific protein had eluted off the column. The antibody was then eluted with 0.2M glycine (pH 2.8) and neutralized with Tris-HCl (pH 8.0). The first two fractions were pooled and the third and fourth fraction were also pooled. The two pooled sets of eluate were dialyzed with PBS overnight. (C) 10 µl of the two pooled sets of elutions were resolved on an SDS-PAGE gel, as were the eluted fractions 5, 10, 15, 20, 25 and 30. A titration of BSA of known concentration was resolved on the right-hand side of the gel. The protein content of the gel was detected by coomassie blue staining. (D) An *in vitro* phosphorylation assay was performed on the ASPP2 fragment (693-1128) using recombinant MAPK in the presence or absence of non-radioactive ATP. 300ng of the recombinant GST-ASPP2 fragment was resolved on an SDS-PAGE gel and western blotted with the purified S-4 phospho-specific antibody. Total ASPP2 (693-1128) content was detected by the 5410 antibody.

4.2.4.2. ASPP2 is phosphorylated *in vivo* by MAPK

Saos2 cells were grown in low serum for 50 hours to remove all background stimulation of Ras, after which the cells were stimulated with epidermal growth factor (EGF) and 20% foetal calf serum (FCS) at various times to activate the endogenous Ras-Raf-MAPK pathway. EGF is known to stimulate MAPK activity via the Ras-Raf pathway in a very rapid and transient manner (Hunter et al., 1985). MAPK stimulation by EGF could be detected using a phosphorylation specific antibody against MAPK; MAPK was seen to be phosphorylated 30 minutes after EGF stimulation (figure 4.5A).

Since endogenous ASPP2 is present at low levels in cells, an immunoprecipitation was performed to facilitate its detection. ASPP2 immunoprecipitations were Western blotted and the phosphorylation state of ASPP2 detected using the purified phospho-specific antibody S-4 against phosphorylated serine 827 mentioned above. One hour after EGF stimulation and 30 minutes after MAPK activation, ASPP2 was found to be phosphorylated *in vivo* (figure 4.5B). The phosphorylation of ASPP2 was rapid and transient as 3 hours after EGF stimulation phosphorylated ASPP2 was barely detectable. An polyclonal antibody against ASPP2, known as bp77, was used as a control to detect the total amount of ASPP2 immunoprecipitated. Although there was some variation in the total amount of ASPP2 detected, transient phosphorylation of ASPP2 was clearly observed (figure 4.5B).

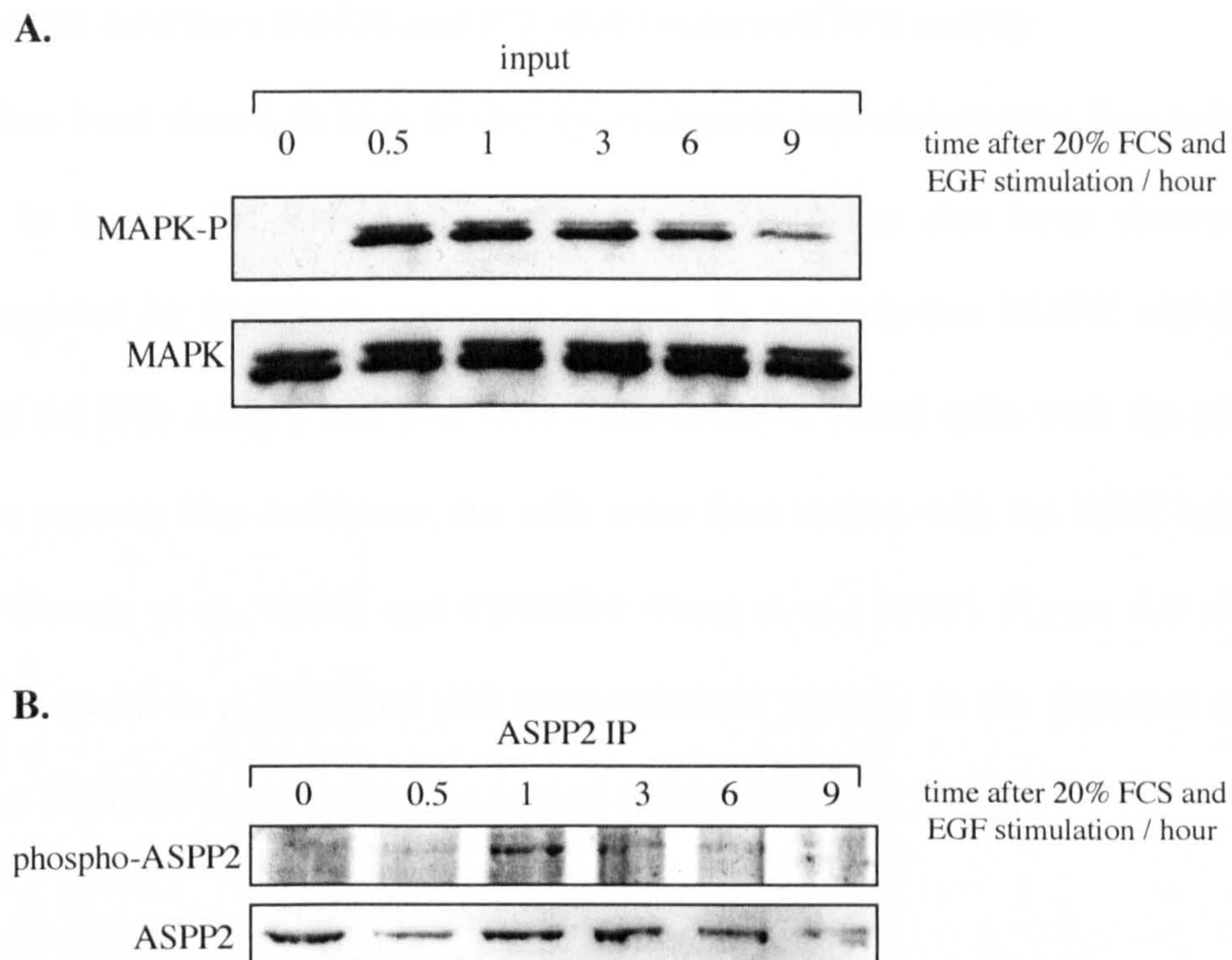


Figure 4.5 *ASPP2 is phosphorylated in vivo by MAPK.* Saos2 cells were starved of serum for 50 hours and then stimulated with 20% FCS and EGF. At the indicated times, cells were harvested and lysates prepared. **(A)** 50 μ g of input protein was resolved on a 15% SDS-PAGE gel. Western blotting was performed with an antibody against phosphorylated MAPK (upper lane). The blot was then reprobed with an antibody against total MAPK (bottom lane). **(B)** 4 mg of lysate was immunoprecipitated with the monoclonal 5410 antibody against ASPP2. The immunoprecipitates were resolved on an 8% SDS-PAGE gel and western blotting was performed with the polyclonal phospho-specific S-4 antibody (upper lane). The blot was then reprobed for total ASPP2 content with the polyclonal bp77 antibody (bottom lane).

4.2.5. Endogenous MAPK activity is necessary for full ASPP activity

4.2.5.1. MEK inhibitors UO126 and PD 98059 reduce ASPP2 activity

ASPP2 has been shown to be activated by oncogenic and endogenous Ras and this is thought to be via the Raf-MAPK pathway. ASPP2 has also been shown to be phosphorylated by MAPK *in vitro* and *in vivo*. To test whether MAPK activity can stimulate ASPP2, ASPP2 and p53 were transfected in Saos2 cells with the p53 pro-apoptotic reporter Bax-luciferase; the cells were then treated with the MEK inhibitors UO126 (Favata et al., 1998) and PD98059 (Pang et al., 1995). Figure 4.6 shows a marked decrease in ASPP2 and p53 transactivation activity in the presence of both — UO126 or PD98059 compared to the control cells treated with DMSO.

The fact that ASPP2 activity is reduced in the absence of endogenous MAPK activity correlates with the previous observation that removal of endogenous Ras by RNAi could reduce ASPP activity. The inhibition of Bax-luciferase activity is consistent with endogenous Ras activating ASPP2 via the MAPK pathway.

A Western blot was performed to check the expression level of transfected ASPP2 and p53. Interestingly, the ASPP2 levels are markedly decreased in the presence of UO126. This will be discussed further in this chapter (section 4.2.5)

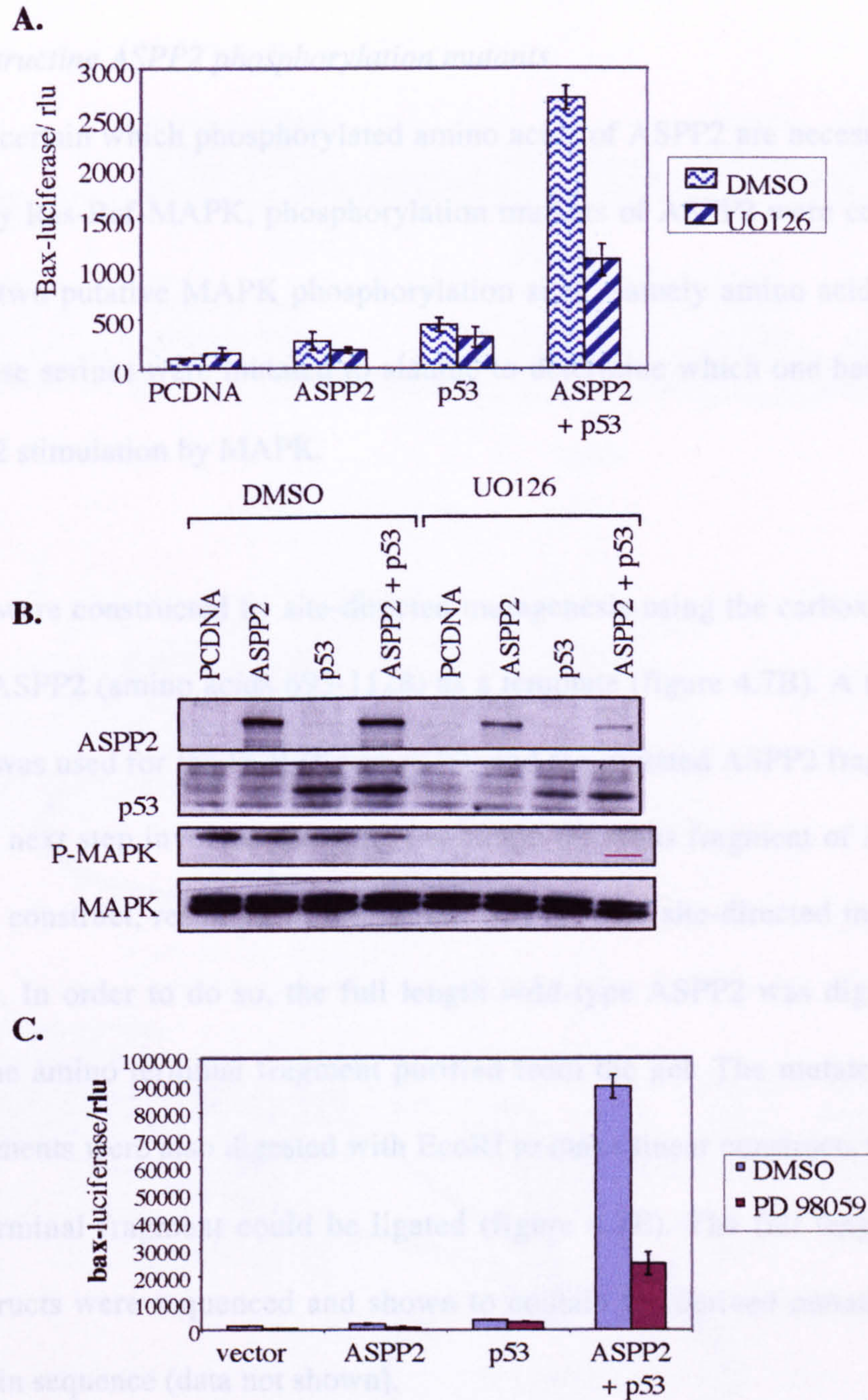


Figure 4.6 *Endogenous MAPK is required for full ASPP2 activity* (A) Saos2 cells were transfected with 4 μ g ASPP2 and 50 ng p53 expression plasmids as indicated, together with 1 μ g bax-luciferase. After transfection, the cells were treated with either 20 μ M UO126 or DMSO as a control for 20 hours. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. The luciferase counts were read and shown as a bar graph. The values shown are means derived from three independent experiments. (B) Aliquots of the luciferase samples were used for western blotting to detect ASPP2 with the V5 antibody, p53 with the DO-1 antibody, phosphorylated MAPK with the phospho-specific MAPK antibody and total MAPK protein as a control. (C) Identical experiment as in A except that Saos2 cells were treated with 100 μ M PD 98059 or DMSO as a control.

4.2.5.2. Constructing ASPP2 phosphorylation mutants

In order to ascertain which phosphorylated amino acids of ASPP2 are necessary for its stimulation by Ras-Raf-MAPK, phosphorylation mutants of ASPP2 were constructed. As there are two putative MAPK phosphorylation sites, namely amino acids 698 and 827, both those serines were mutated to alanine to determine which one had a crucial role in ASPP2 stimulation by MAPK.

The mutants were constructed by site-directed mutagenesis using the carboxy-terminal fragment of ASPP2 (amino acids 693-1128) as a template (figure 4.7B). A titration of the template was used for the PCR (figure 4.7D) and the mutated ASPP2 fragment was purified. The next step involved inserting the amino terminus fragment of ASPP2 (1-692) into the construct, resulting in full length ASPP2 with site-directed mutagenesis (figure 4.7C). In order to do so, the full length wild-type ASPP2 was digested with EcoRI and the amino terminal fragment purified from the gel. The mutated carboxy terminal fragments were also digested with EcoRI to make linear constructs into which the amino terminal fragment could be ligated (figure 4.7E). The full length ASPP2 mutant constructs were sequenced and shown to contain the derived mutation but no other change in sequence (data not shown).

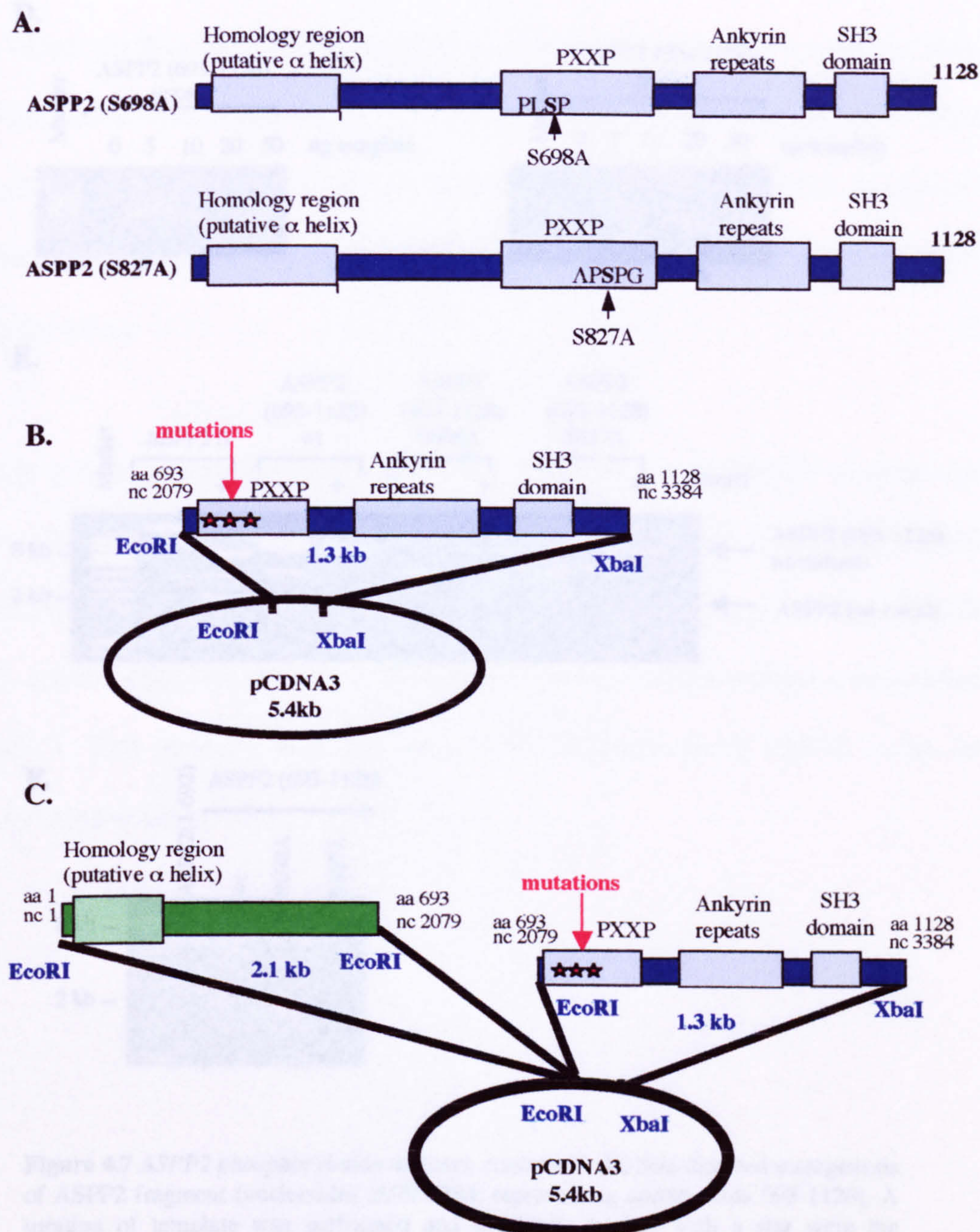
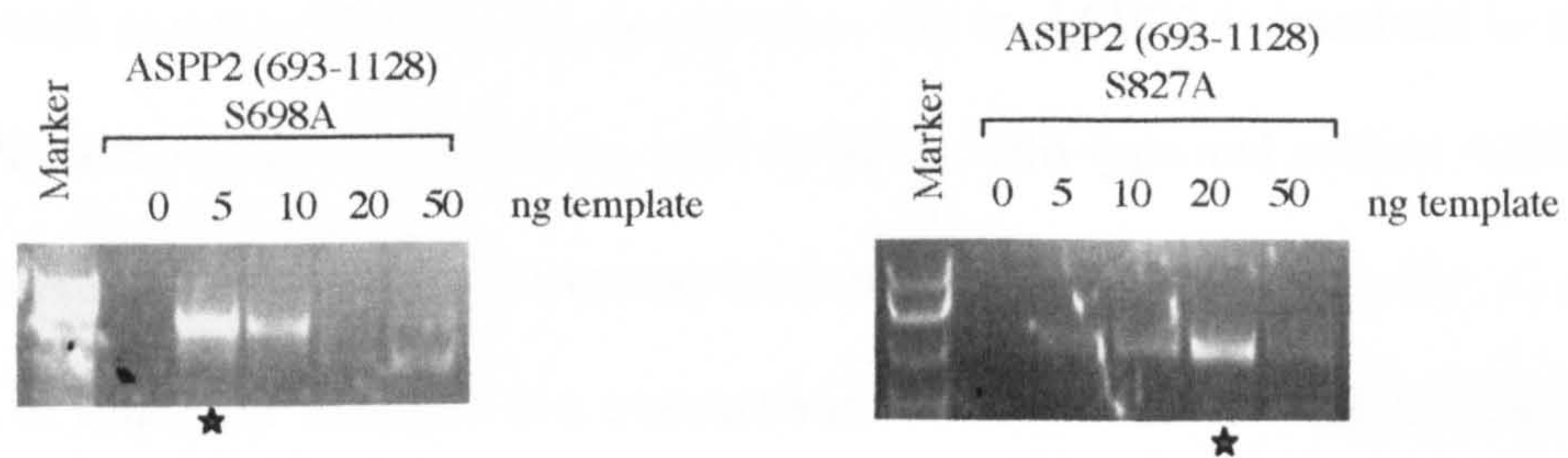
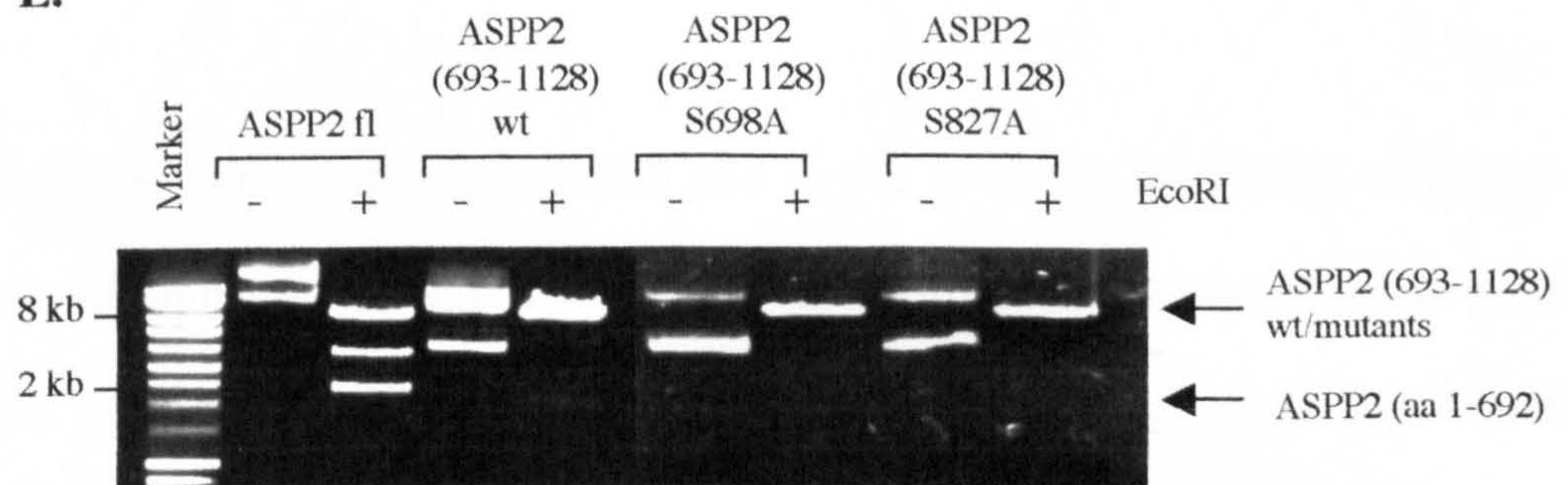


Figure 4.7 Construction of plasmids expressing ASPP2 phosphorylation mutants. (A) Schematic representation of the construction of ASPP2 mutants expression plasmids. The homologous region in the amino terminus is shown, as is the proline rich region (PXXP), the ankyrin repeats and the SH3 domain. The two putative MAPK phosphorylation sites are shown (serine 698 and serine 827). Both of these putative phosphorylation sites were mutated to alanine as described in Materials & Methods. (B) The mutations were introduced by site-directed mutagenesis in the ASPP2 fragment representing amino acids (aa) 693-1128 and nucleotides (nc) 2079-3384. (C) Schematic representation of the ASPP2 amino-terminus fragment (amino acids 1-693, representing nucleotides 1-2079) subsequently added to the mutated carboxy-terminus fragment.

D.



E.



F.

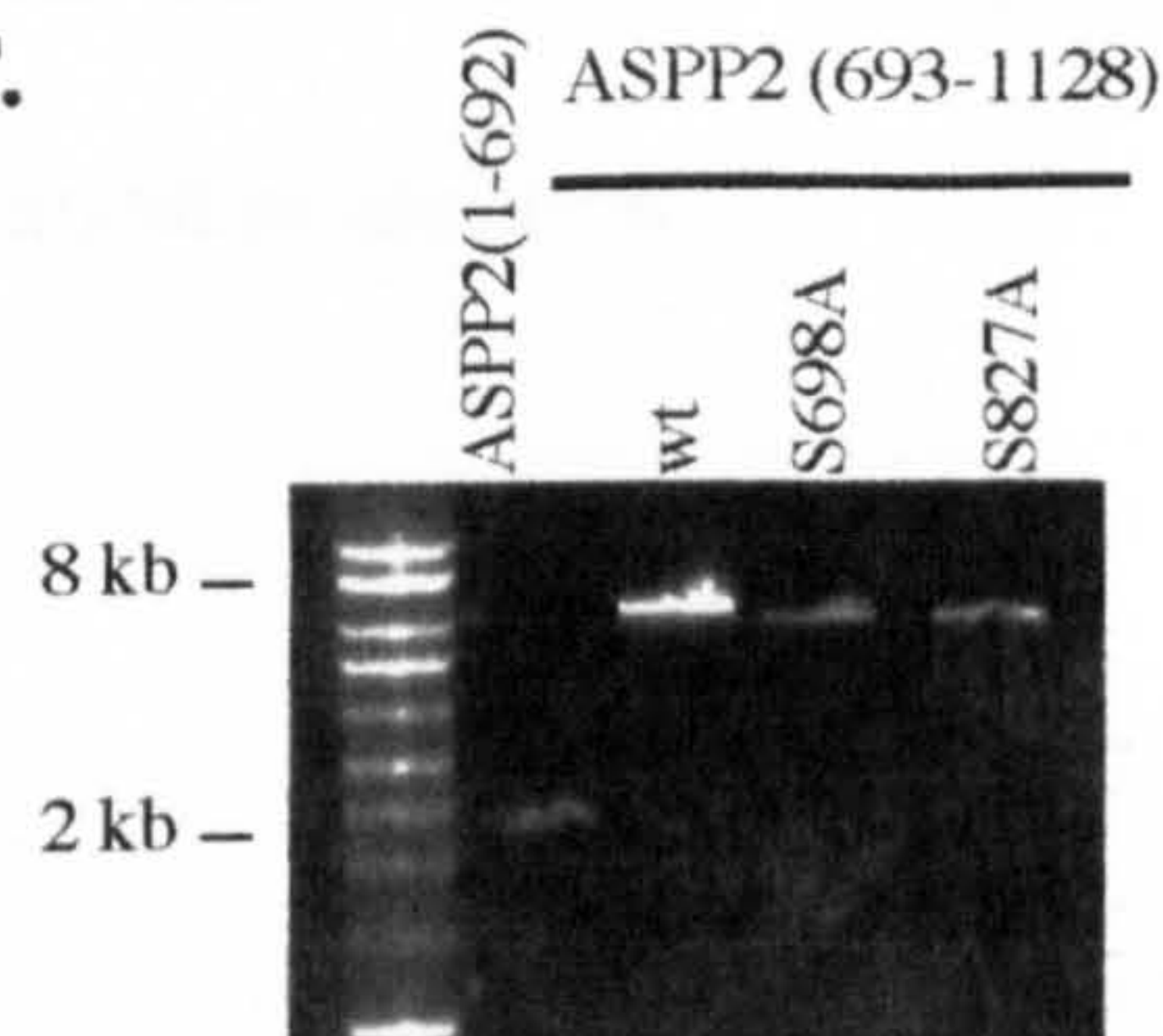


Figure 4.7 *ASPP2 phosphorylation mutants, continued* (D) Site-directed mutagenesis of ASPP2 fragment (nucleotides 2079-3384, representing amino acids 693-1128). A titration of template was performed and the lanes marked with a star were the fragments chosen to continue the site-directed mutagenesis. (E) Full length ASPP2 was digested with EcoRI as were the mutated and wild-type ASPP2 fragments (nucleotides 2079-3384, representing amino acids 693-1128). All digests were resolved on a 1% agarose gel. The digested fragment of ASPP2 representing the nucleotides 1-2079 (amino acids 1-693) ran at 2kb and was purified from the gel. The digested bands representing the wild-type and mutants fragments (2079-3384 nucleotides, 693-1128 amino acids) ran as a single band at 8 kb and were also purified. (F) An aliquot of the purified fragments was resolved on a gel to check the efficacy of the purification. The amino-terminal fragment of ASPP2 was then ligated into the carboxy-terminal wild-type and mutant fragments of ASPP2 to produce full length wild-type and mutant ASPP2.

4.2.5.3. RafCX stimulation of ASPP2 is via ASPP2 phosphorylation at serine 827

To test which putative MAPK phosphorylation site in ASPP2 is involved in the Ras-Raf-MAPK stimulation of ASPP2, the activity of the wild-type and mutant ASPP2 were compared. ASPP2 wild-type and mutants were transfected into Saos2 cells; all mutants were able to synergize with p53 in a transactivation assay as effectively as ASPP2 wild-type (figure 4.8A).

When Raf CX was co-transfected with p53 in the presence of ASPP2 wild-type or mutants a difference was observed with the ASPP2 (S827A) mutant. Whereas ASPP2 wild-type, and ASPP2 (S698) activity was increased 2.5 fold in the presence of RafCX and p53, ASPP2 (S827A) did not stimulate p53 activity further when co-transfected with RafCX. This strongly suggests that ASPP2 stimulation by RafCX is via its phosphorylation at serine 827.

4.2.5.4. K-RasV12 stimulation of ASPP1 is via ASPP1 phosphorylation on Serine 746

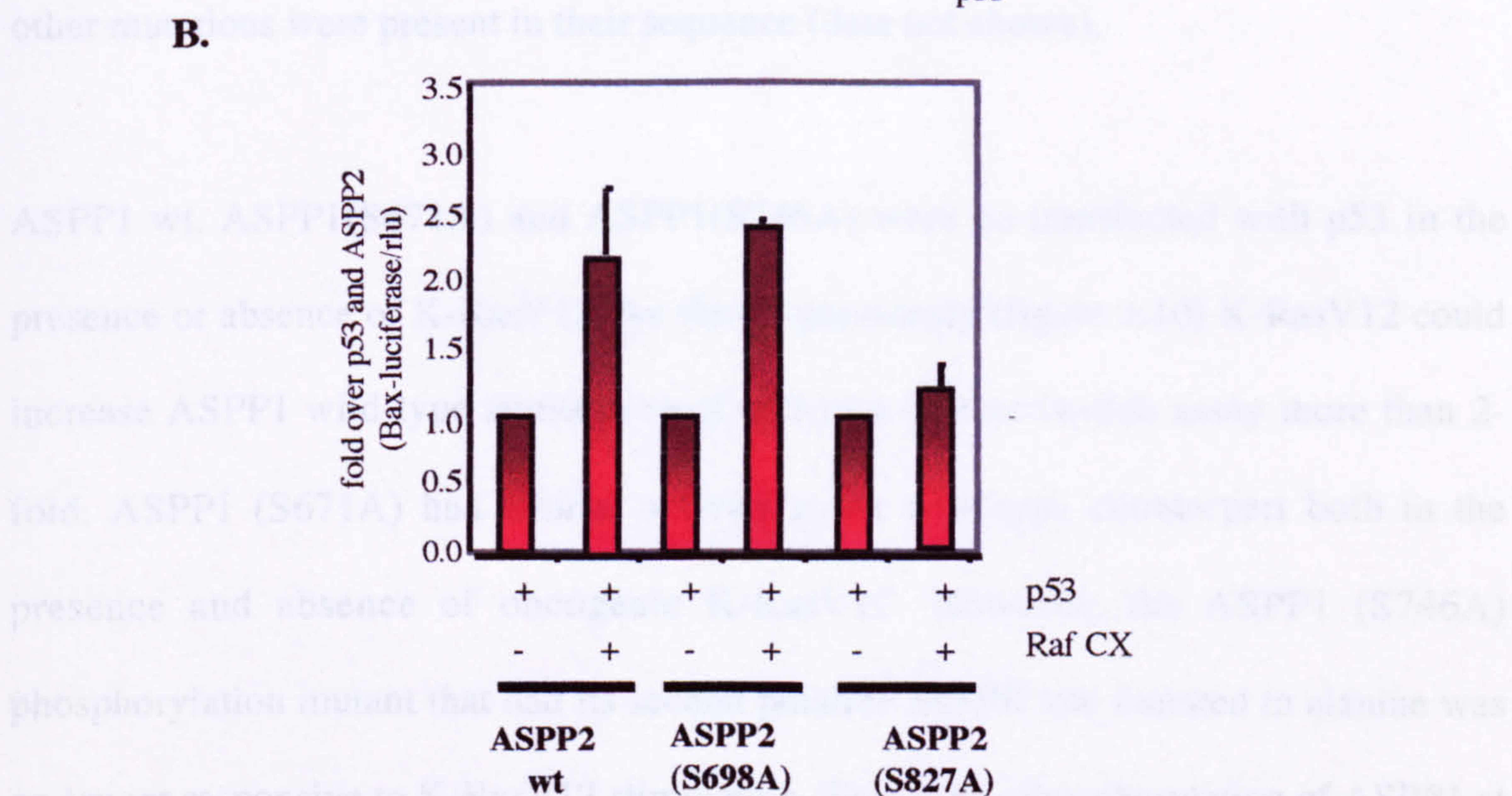
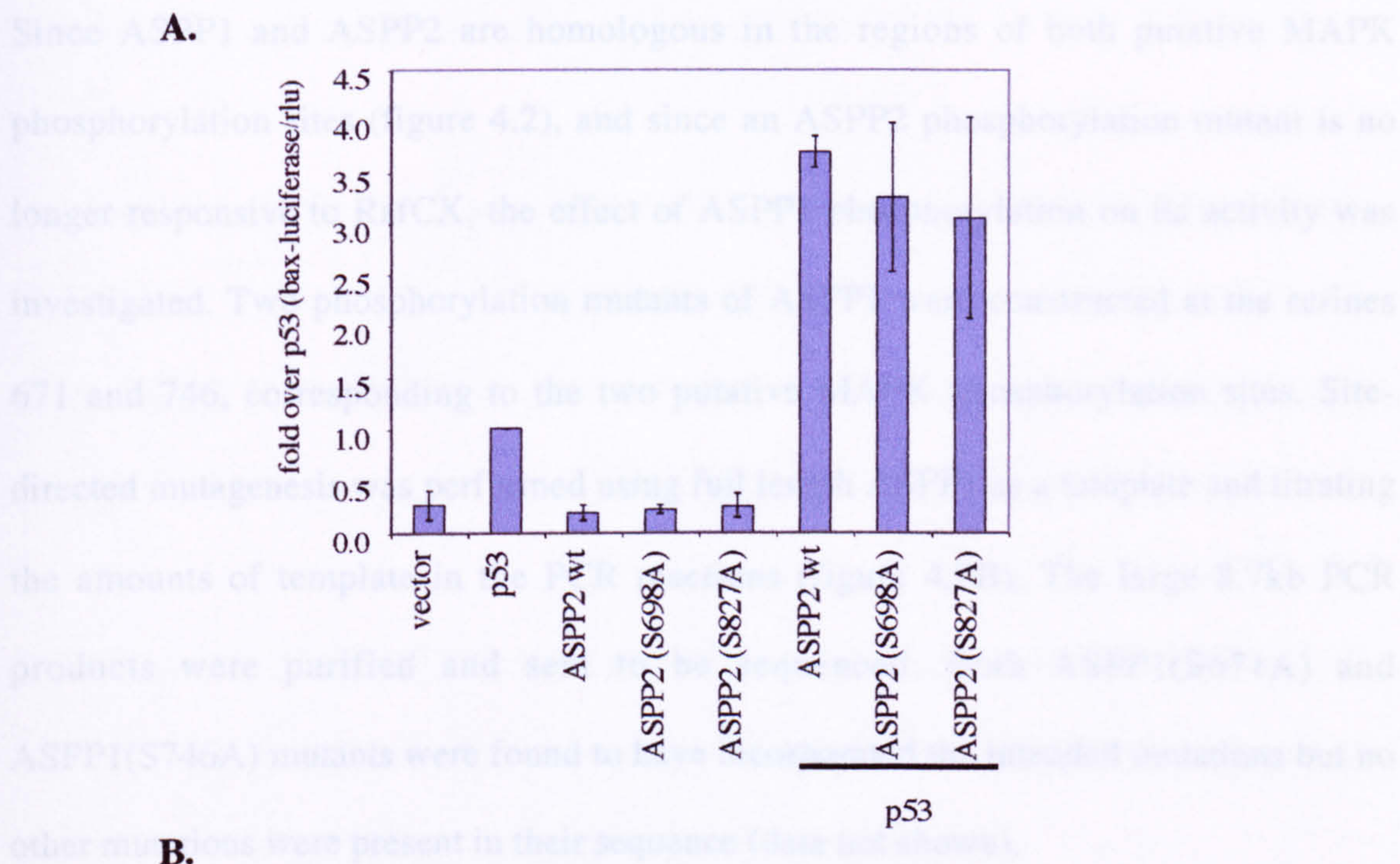


Figure 4.8 ASPP2 (S827A) does not respond to Raf CX stimulation (A) Saos2 cells were transfected 4 μ g of ASPP2 wild-type, ASPP2(S698A) or ASPP2(S827A) in the presence or absence of 50 ng p53, as indicated. All cells were co-transfected with 1 μ g Bax-luciferase. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. (B) The same experiment as A was performed with 1.5 μ g RafCX co-transfected as indicated. The luciferase value of ASPP2 and p53 were taken as one to see the fold increase by RafCX. The mean values shown are derived from three independent experiments.

4.2.5.4. K-RasV12 stimulation of ASPP1 is via ASPP1 phosphorylation on serine 746

Since ASPP1 and ASPP2 are homologous in the regions of both putative MAPK phosphorylation sites (figure 4.2), and since an ASPP2 phosphorylation mutant is no longer responsive to RafCX, the effect of ASPP1 phosphorylation on its activity was investigated. Two phosphorylation mutants of ASPP1 were constructed at the serines 671 and 746, corresponding to the two putative MAPK phosphorylation sites. Site-directed mutagenesis was performed using full length ASPP1 as a template and titrating the amounts of template in the PCR reactions (figure 4.9B). The large 8.7kb PCR products were purified and sent to be sequenced. Both ASPP1(S671A) and ASPP1(S746A) mutants were found to have incorporated the intended mutations but no other mutations were present in their sequence (data not shown).

ASPP1 wt, ASPP1(S671A) and ASPP1(S746A) were co-transfected with p53 in the presence or absence of K-RasV12. As shown previously (figure 3.10) K-RasV12 could increase ASPP1 wild-type stimulation of p53 in a transactivation assay more than 2-fold. ASPP1 (S671A) had similar activity to its wild-type counterpart both in the presence and absence of oncogenic K-RasV12. However, the ASPP1 (S746A) phosphorylation mutant that had its second putative MAPK site mutated to alanine was no longer responsive to K-RasV12 stimulation. Therefore, phosphorylation of ASPP1 at amino acid 746 is necessary for its stimulation by K-RasV12.

Aliquots of the transactivation assay lysates were used to perform a Western blot to detect expression levels of transfected ASPP1, p53 and K-RasV12 (figure 4.9D). ASPP1 and wild-type and mutants were all expressed at similar levels. This confirms

that the difference in the response of the ASPP1 (S747A) to Ras is due to the difference in its intrinsic activity rather than its expression levels.

Interestingly, ASPP1 wild-type and ASPP1 (S671A) levels were increased in the presence of K-RasV12, whereas ASPP1 (S746A) levels were not affected by co-expression of K-RasV12. This point will be pursued further in the latter parts of this chapter.

Thus, both ASPP1 and ASPP2 have a MAPK phosphorylation site, at amino acids 746 and 827 respectively, that needs to be phosphorylated for full stimulation of the proteins by the Ras-Raf-MAPK signalling pathway.

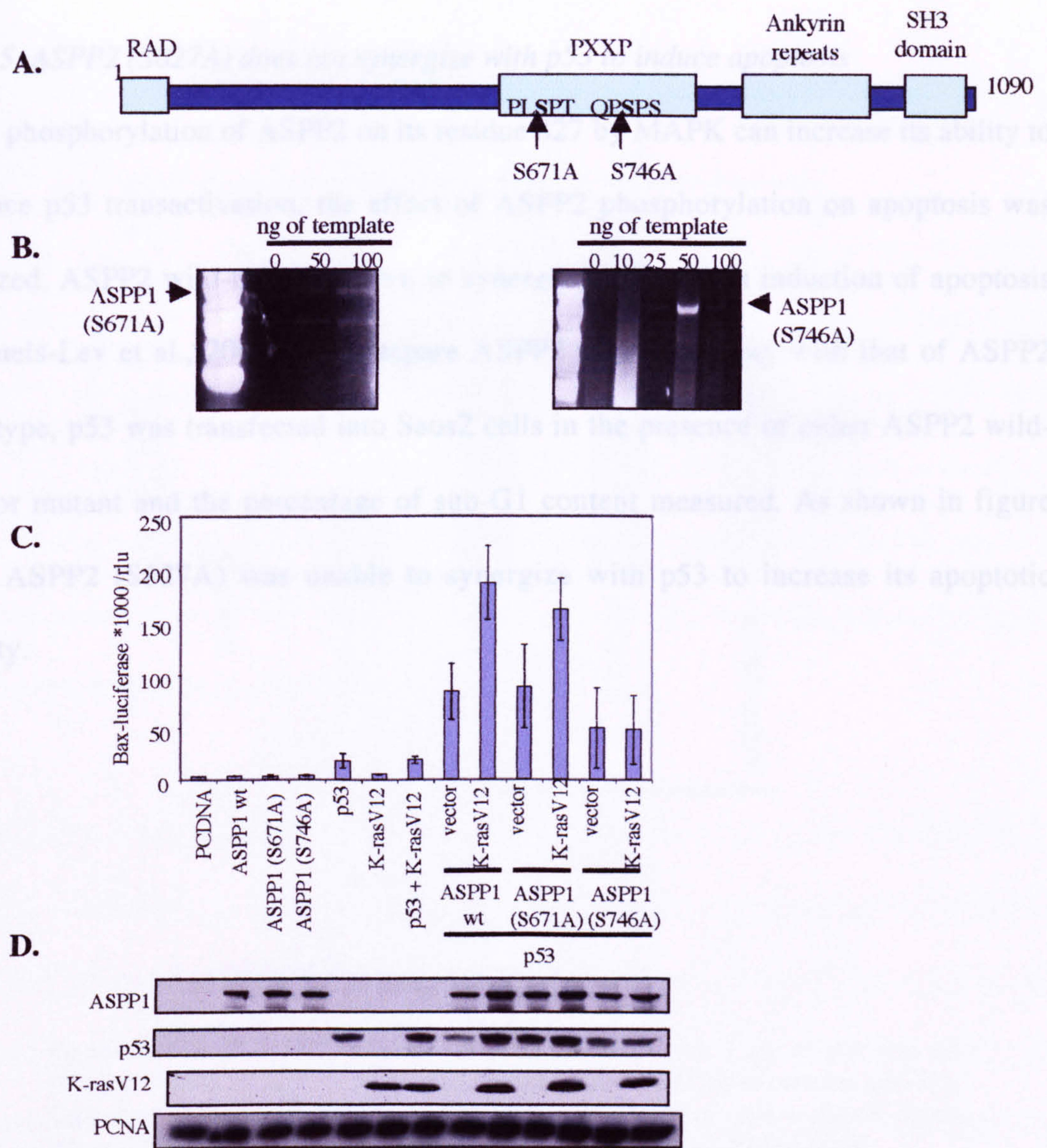


Figure 4.9 *ASPP1 phosphorylation mutant S746A does not respond to K-rasV12 stimulation* (A) The two putative MAPK phosphorylation sites at amino acids 671 and 746 were mutated to alanine by oligonucleotide-directed mutagenesis, using the cDNA as a template. The Ras association domain (RA), proline rich region (PXXP), ankyring repeats and SH3 domain are shown as indicated. (B) Full length ASPP1 was used as a template for PCR with primers containing the desired mutation. The left panel shows the PCR of ASPP1(S671A) and the right panel shows the PCR of the ASPP1(S746A) mutant. The arrows indicate the band of interest which was purified. The mutants were subsequently sequenced. (C) Saos2 cells were transfected with 4 μ g of wild-type ASPP1 or phosphorylation mutants ASPP1 in the presence of 50 ng of p53 and 1.5 μ g K-rasV12 as indicated. All samples were co-transfected with Bax-luciferase. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. The luciferase counts are shown as a bar graph. The mean values are derived from three independent experiments. (D) An aliquot of each luciferase lysate was used for western blotting which was probed with V5 antibody against ASPP1, DO-1 antibody against p53, HA antibody against K-rasV12 and PC-10 against PCNA.

4.2.5.5. ASPP2 (S827A) does not synergize with p53 to induce apoptosis

Since phosphorylation of ASPP2 on its residue 827 by MAPK can increase its ability to enhance p53 transactivation, the effect of ASPP2 phosphorylation on apoptosis was analyzed. ASPP2 wild-type is known to synergize with p53 in induction of apoptosis (Samuels-Lev et al., 2001). To compare ASPP2 mutant activity with that of ASPP2 wild-type, p53 was transfected into Saos2 cells in the presence of either ASPP2 wild-type or mutant and the percentage of sub-G1 content measured. As shown in figure 4.10, ASPP2 (S827A) was unable to synergize with p53 to increase its apoptotic activity.

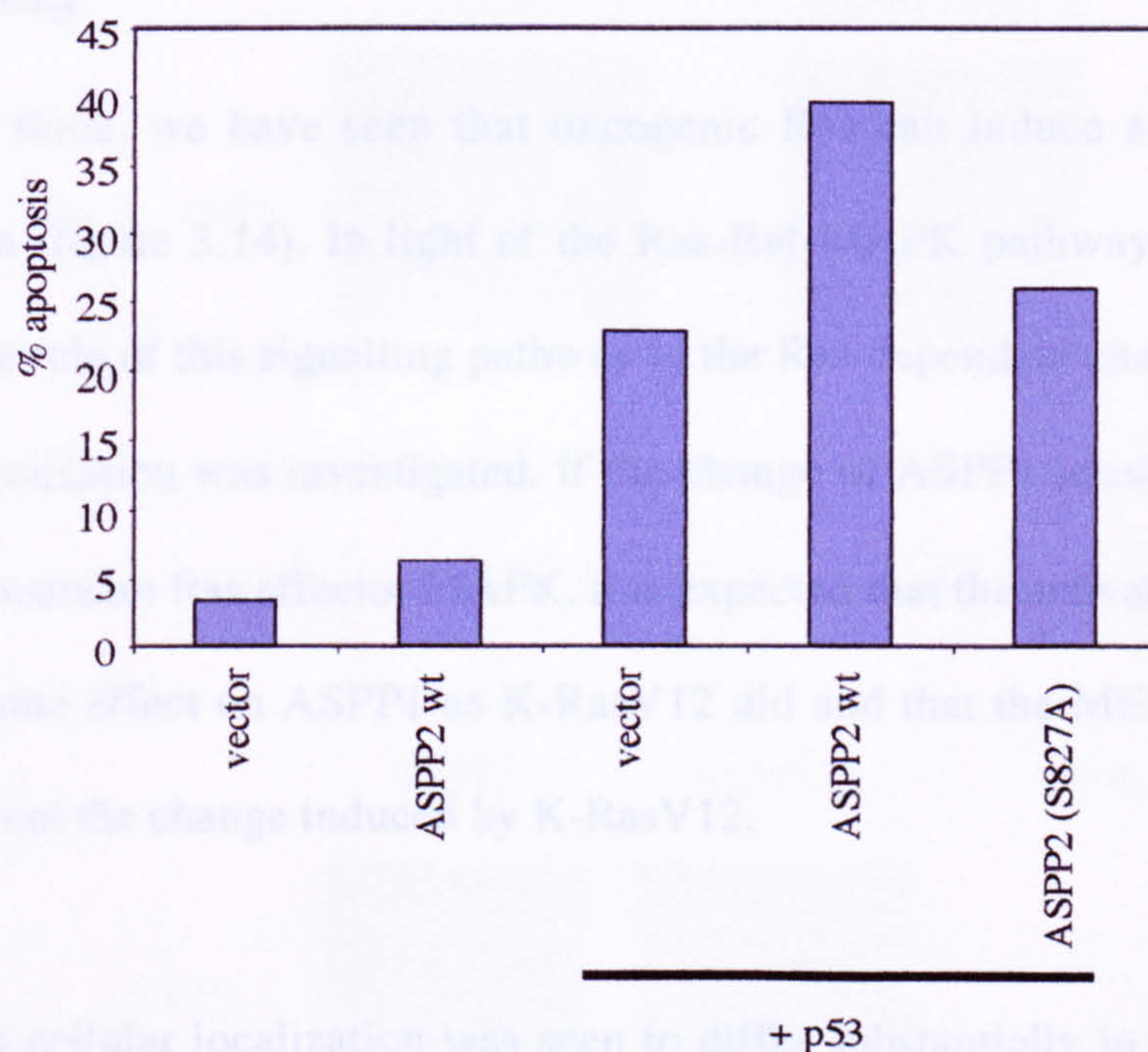


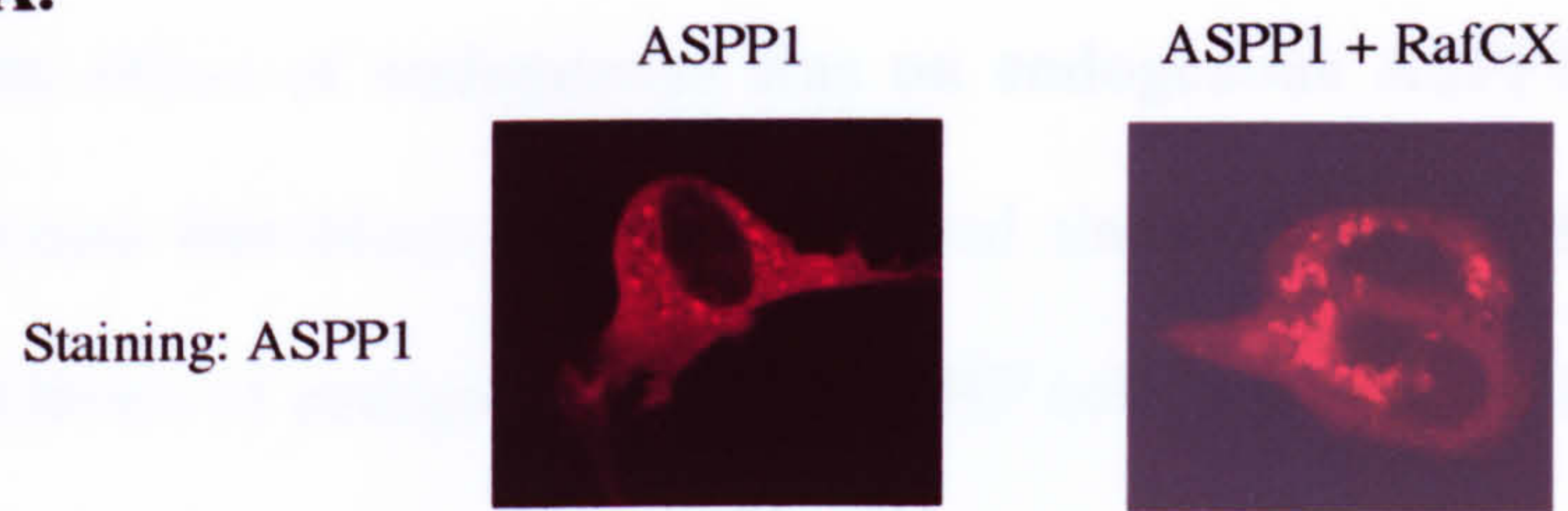
Figure 4.10 *ASPP2 (S827A) does not synergize with p53.* 1 μ g of p53 was co-transfected with 10 μ g of ASPP2 wild-type or ASPP2 (S827A) expression plasmids. Cells were collected for FACS analysis and the percentage of cells in sub-G1 shown above. This experiment was done in collaboration with Daniele Bergamaschi.

4.2.6. K-RasV12-dependent change of ASPP1 sub-cellular localization is mediated by MAPK activity

In chapter three, we have seen that oncogenic Ras can induce a change in ASPP1 localization (figure 3.14). In light of the Ras-Raf-MAPK pathway stimulating ASPP activity, the role of this signalling pathway in the Ras-dependent change of ASPP1 sub-cellular localization was investigated. If the change of ASPP1 localization is mediated by the downstream Ras effector MAPK, it is expected that the activated Raf-CAAX will have the same effect on ASPP1 as K-RasV12 did and that the MEK inhibitor UO126 would prevent the change induced by K-RasV12.

ASPP1 sub-cellular localization was seen to differ substantially in the presence of K-RasV12, resulting in globular doughnut-like shapes in the cytoplasm after K-RasV12 co-transfection compared of the usual diffuse pattern ASPP1. Raf-CAAX co-transfection with ASPP1 resulted in an identical change of localization of ASPP1 as seen with co-transfection with K-RasV12. When cells co-transfected with K-RasV12 and ASPP1 were treated with the MEK inhibitor UO126, the ASPP1 sub-cellular localization reverted back to that seen with ASPP1 transfected alone, in the absence of K-RasV12 (figure 4.11). Therefore, the K-RasV12-dependent change in ASPP1 localization is mediated via the MAPK activity.

A.



B.

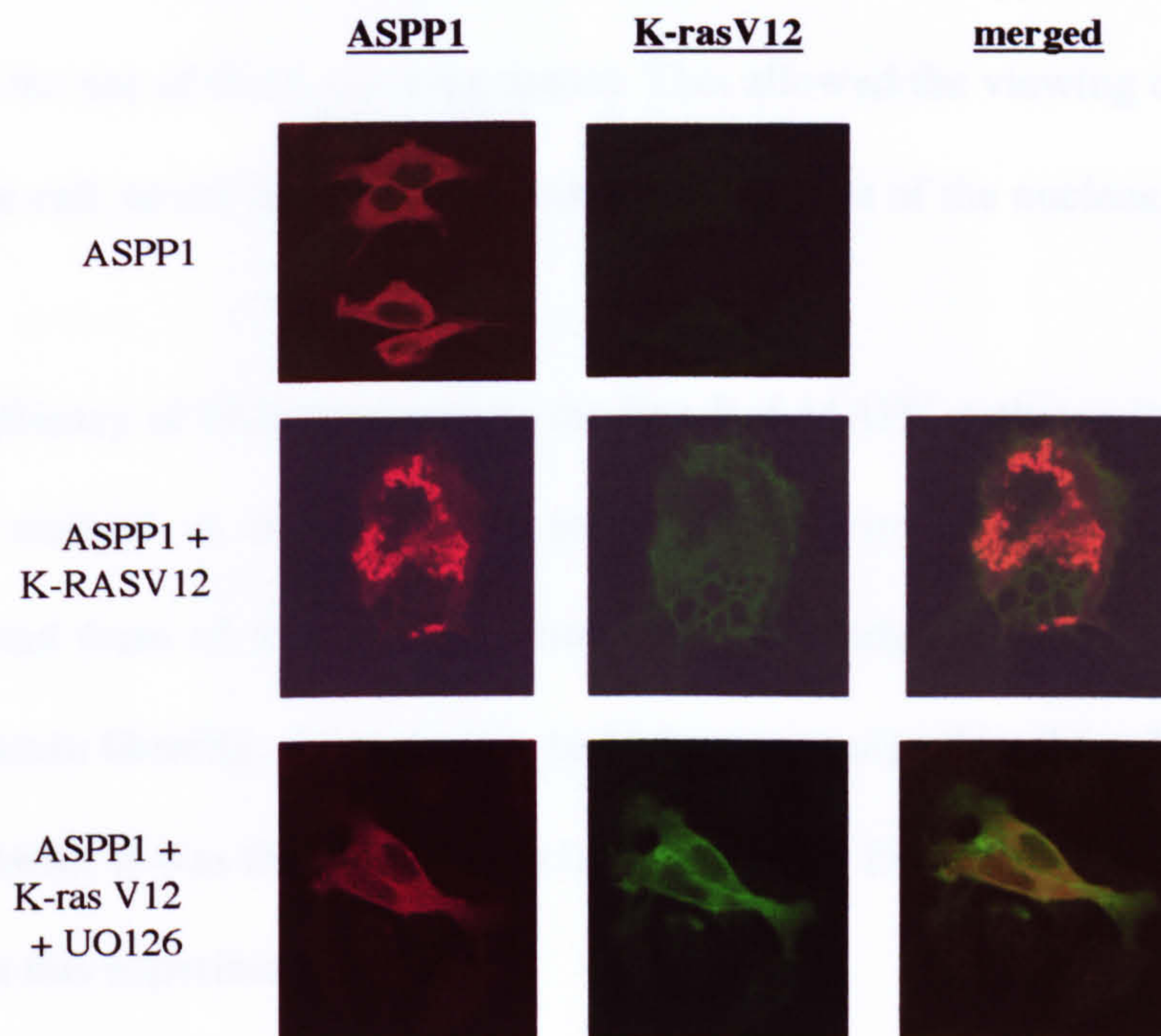


Figure 4.11 *K-rasV12 effect on ASPP1 change of cellular localization is dependent on MAPK activity.* (A) U2OS cells were transfected with ASPP1 alone (left panel) or ASPP1 and activated RafCX (right panel). The cells were stained with the V5 antibody to detect ASPP1. (B) U2OS cells were transfected with ASPP1 alone or ASPP1 and K-rasV12 in the presence or absence of 20 μ M of the MAPK inhibitor UO126. The cells were fixed and double-stained with the V5 monoclonal mouse antibody (ASPP1) and the Ras monoclonal rat antibody 259. ASPP1 was detected with TRITC and ras with FITC. The cells were visualized by confocal microscopy.

4.2.7. Endogenous ASPP1 does not change localization after EGF stimulation

To test the effect of endogenous Ras on endogenous ASPP1 cellular localization, normal human fibroblasts (NHF) were used since primary cells have been shown to have high levels of endogenous ASPP1. NHF cells were grown in low serum overnight to remove background stimulation of endogenous Ras and then stimulated with EGF for 0, 15, 30 and 60 minutes. After the indicated times the cells were fixed and stained with the ASPP1 antibody LX054.2 which is specific to ASPP1 and recognizes the epitope in the amino-terminus of ASPP1 (figure 4.12). Confocal microscopy was used to visualize ASPP1 with the use of the Z-stacking option. This allowed the viewing of a 0.2 μ m thick section of the cell, to see any possible shuttling in and out of the nucleus.

To test the efficacy of EGF to stimulate the Ras-Raf-MAPK pathway in these cells, the cells were stained at the different time points with an antibody against the phosphorylated form of MAPK. Unfortunately, the antibody used, although working well for Western blotting, did not seem to pick up any signal in the cell staining assay (data not shown). It was therefore inconclusive whether EGF was able to stimulate Ras effectively in this experiment.

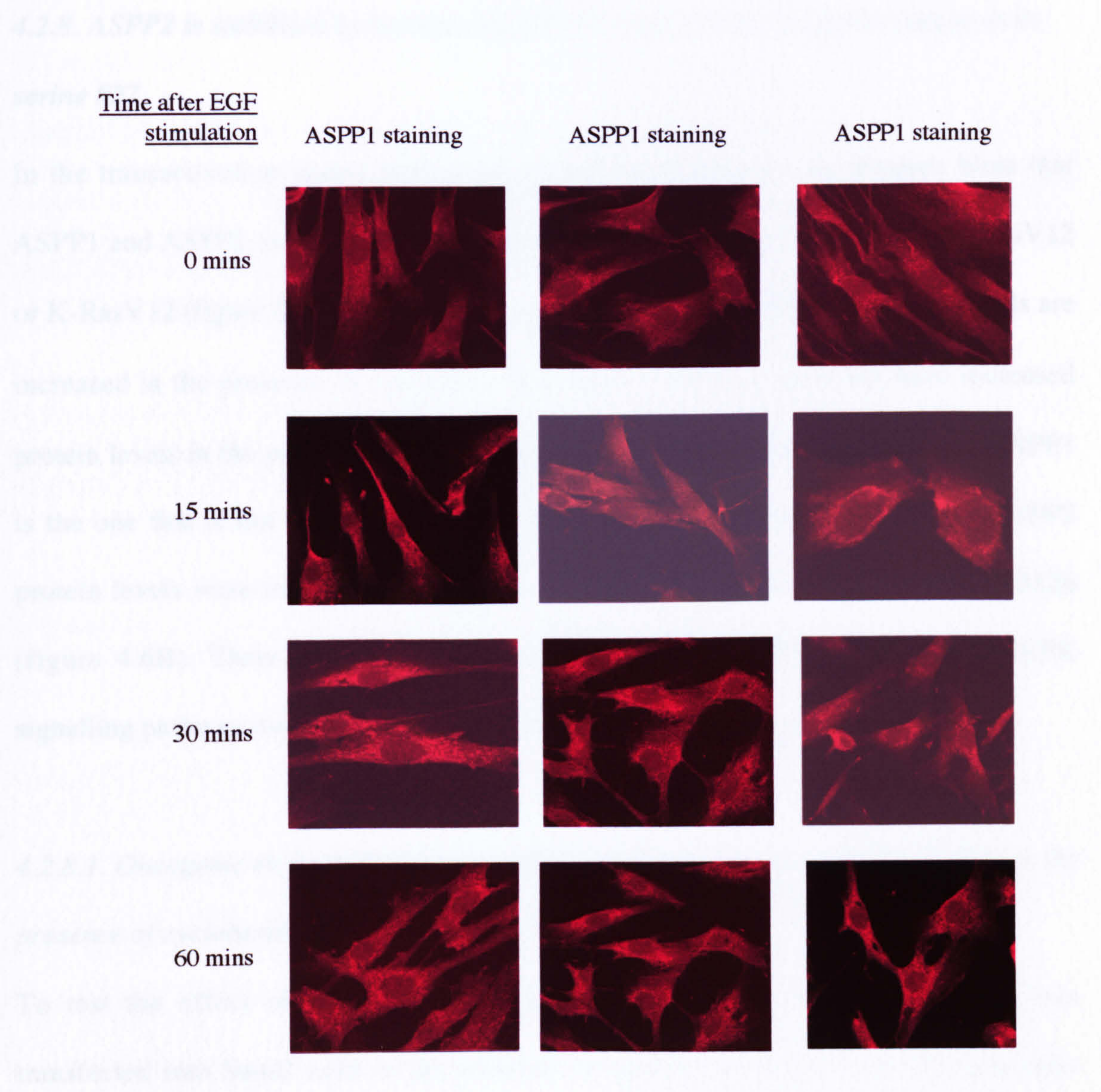


Figure 4.12. *ASPP1 does not change its localization in Normal Human Fibroblasts after EGF stimulation.* Normal human fibroblasts (NHF) cells were starved of serum overnight and then stimulated with EGF for the time points indication. At the indicated time, the cells were fixed and stained with LX054.2 against endogenous ASPP1. The three columns represent three different stainings for ASPP1. The cells were visualized by confocal microscopy.

4.2.8. ASPP2 is stabilized by the Ras-Raf-MAPK pathway via phosphorylation at its serine 827

In the transactivation assays performed so far, it is noticeable in Western blots that ASPP1 and ASPP2 expression levels increased in the presence of oncogenic H-RasV12 or K-RasV12 (figure 3.10B). Interestingly, although ASPP1 wild-type protein levels are increased in the presence of oncogenic Ras, ASPP1 (S746A) does not have increased protein levels in the presence of K-RasV12 (figure 4.9D). This mutant form of ASPP1 is the one that is not stimulated by oncogenic Ras. It was also observed that ASPP2 protein levels were markedly decreased in the presence of the MEK inhibitor UO126 (figure 4.6B). These observations led to the hypothesis that the Ras-Raf-MAPK signalling pathway might stimulate ASPP activity by increasing its protein stability.

4.2.8.1. Oncogenic H-RasV12 stabilizes ASPP2 wild-type but not ASPP2 (S827A) in the presence of cycloheximide

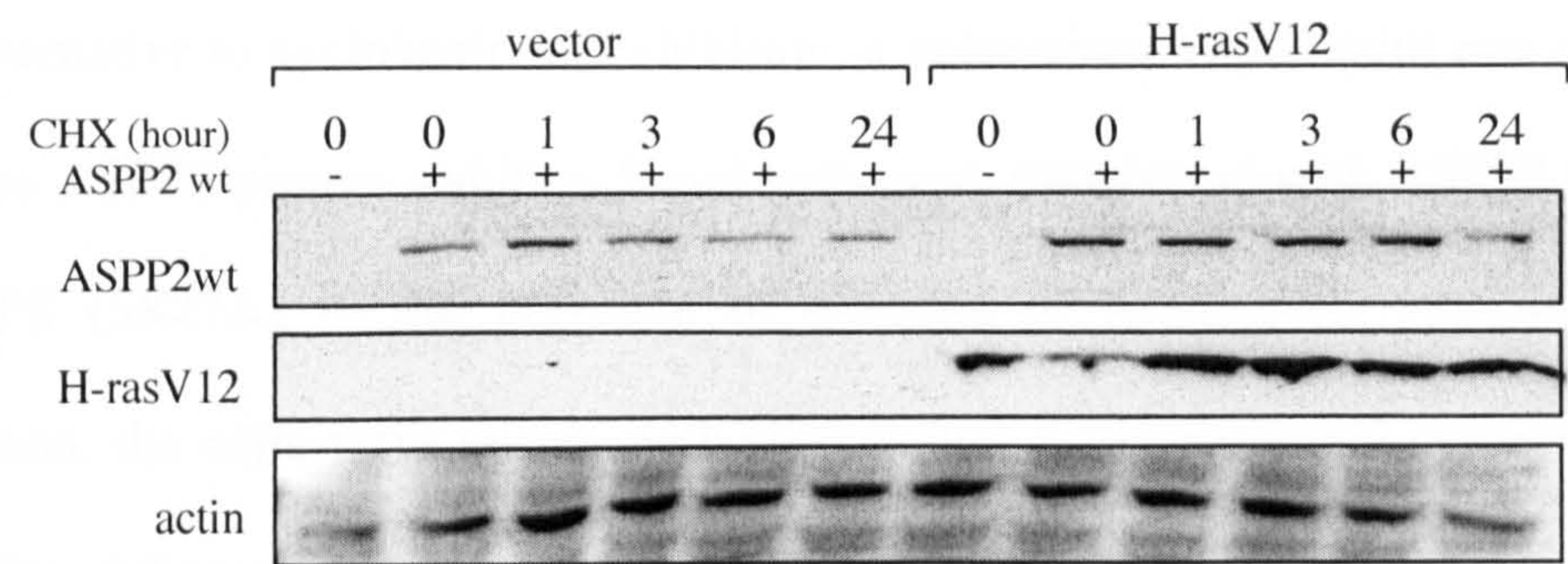
To test the effect of oncogenic Ras on ASPP2 stability, ASPP2 wild-type was transfected into Saos2 cells in the presence or absence of H-RasV12. 16 hours after transfection, protein synthesis was inhibited by the drug cycloheximide. Lysates were collected at various time points after cycloheximide addition and the levels of ASPP2 were detected by Western blotting.

In the absence of H-RasV12, ASPP2 levels were seen to be significantly reduced by 6 hours, suggesting a short half life. However, in the presence of H-RasV12, the stability of ASPP2 seemed to be increased as the levels of ASPP2 protein were similar 6 hours after cycloheximide addition to those at time 0 of cycloheximide addition (figure 4.13A). This suggests that H-RasV12 could increase the half-life of wild-type ASPP2.

When the ASPP2 (S827A) mutant that can no longer be phosphorylated by MAPK was transfected, a similar rate of degradation was seen compared to wild-type ASPP2. Unlike wild-type ASPP2, however, H-RasV12 did not seem to stabilize the ASPP (S827A) protein levels (figure 4.13B).

It can therefore be concluded that H-RasV12 increases ASPP2 wild-type stability and does so via MAPK phosphorylation of the ASPP2 serine 827.

A.



B.

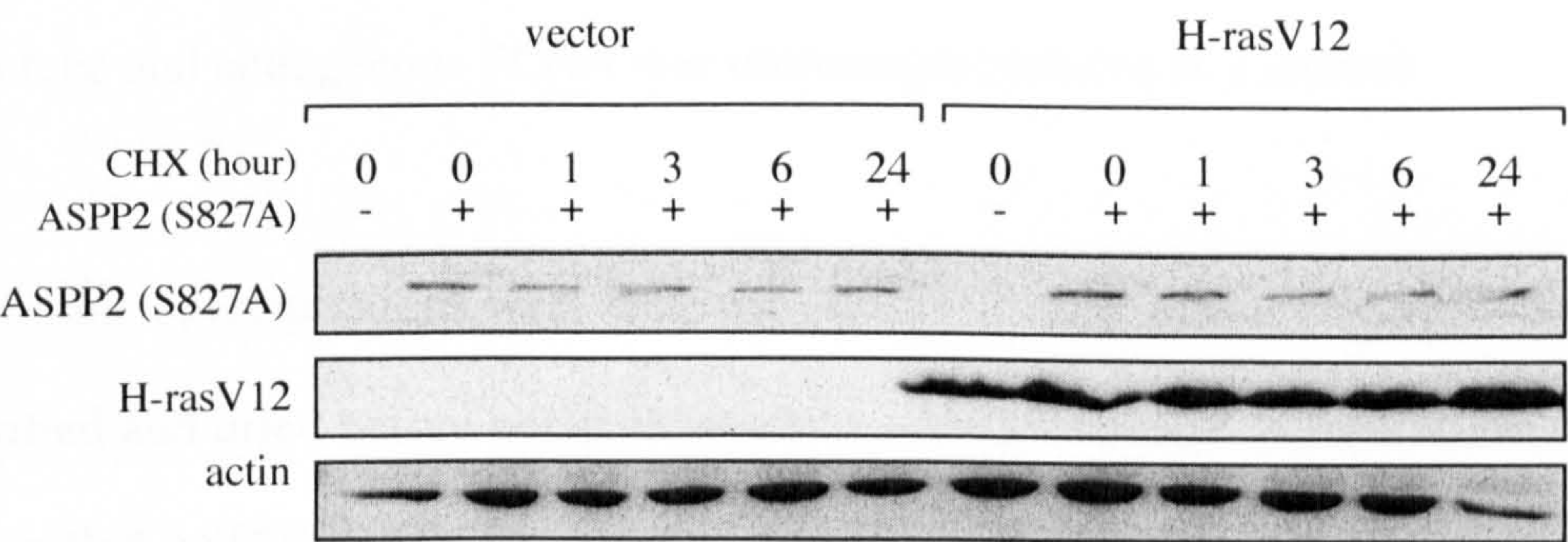


Figure 4.13 *H-rasV12 stabilizes ASPP2 wt but not ASPP2 (S827A)* . Saos2 cells were transfected with either ASPP2 wild-type (**A**) or mutant ASPP2 (S827A) expression plasmids (**B**) in the presence or absence of co-transfected H-RasV12. 16 hours after transfection, 50 µg/ml cycloheximide was added to the cells for the time indicated. 30 µg of the lysates were resolved on an SDS-PAGE gel and western blotting was performed with the V5 antibody to detect transfected ASPP2 and the HA antibody to detect transfected H-rasV12. Anti-actin antibody was used as a loading control.

4.2.8.2. Pulse chase analysis shows that oncogenic H-RasV12 increases ASPP2 wild-type protein levels but not those of ASPP2 (S827A)

As an alternative to cycloheximide inhibition, a pulse-chase experiment was conducted to analyse ASPP2 protein stability. Saos2 cells were transfected with ASPP2 wild-type or ASPP2 (S827A) in the presence or absence of K-RasV12. 12 hours after transfection, the cells were pulsed with ³⁵S-labelled methionine and cysteine for two hours. The radioactive medium was then washed off and replaced with unlabelled medium. The cells were lysed at 0, 6 and 24 hours after the radioactive medium was removed. An immunoprecipitation was performed for transfected ASPP2 using the V5 antibody. After the ASPP2 immunoprecipitation, the supernatants were transferred to a fresh tube and endogenous PCNA was immunoprecipitated as a control.

The immunoprecipitations were resolved on SDS-PAGE gels and the gels were fixed, amplified and dried before being exposed to a phosphoimager screen. Detection of the ³⁵S-labelled ASPP2 and PCNA are shown in figure 4.14A. The stability of ASPP2 wild-type and ASPP2 (S827A) mutant was similar when transfected alone. When K-RasV12 was co-transfected with ASPP2 wild-type, there were much higher levels of ASPP2 wild-type protein expression at time 0 compared to cells transfected with ASPP2 alone. Quantification of the intensity of the bands showed that there was almost twice as much ASPP2 protein when co-transfected with K-RasV12 at time 0 compared to ASPP2 alone at time 0 (figure 4.14B). The protein levels of ASPP2 in the presence of K-RasV12 at times 6 and 24 hours were similar to those of ASPP2 in the absence of K-RasV12. Unlike ASPP2 wild-type, ASPP2 (S827A) protein levels were not affected by the presence of K-RasV12.

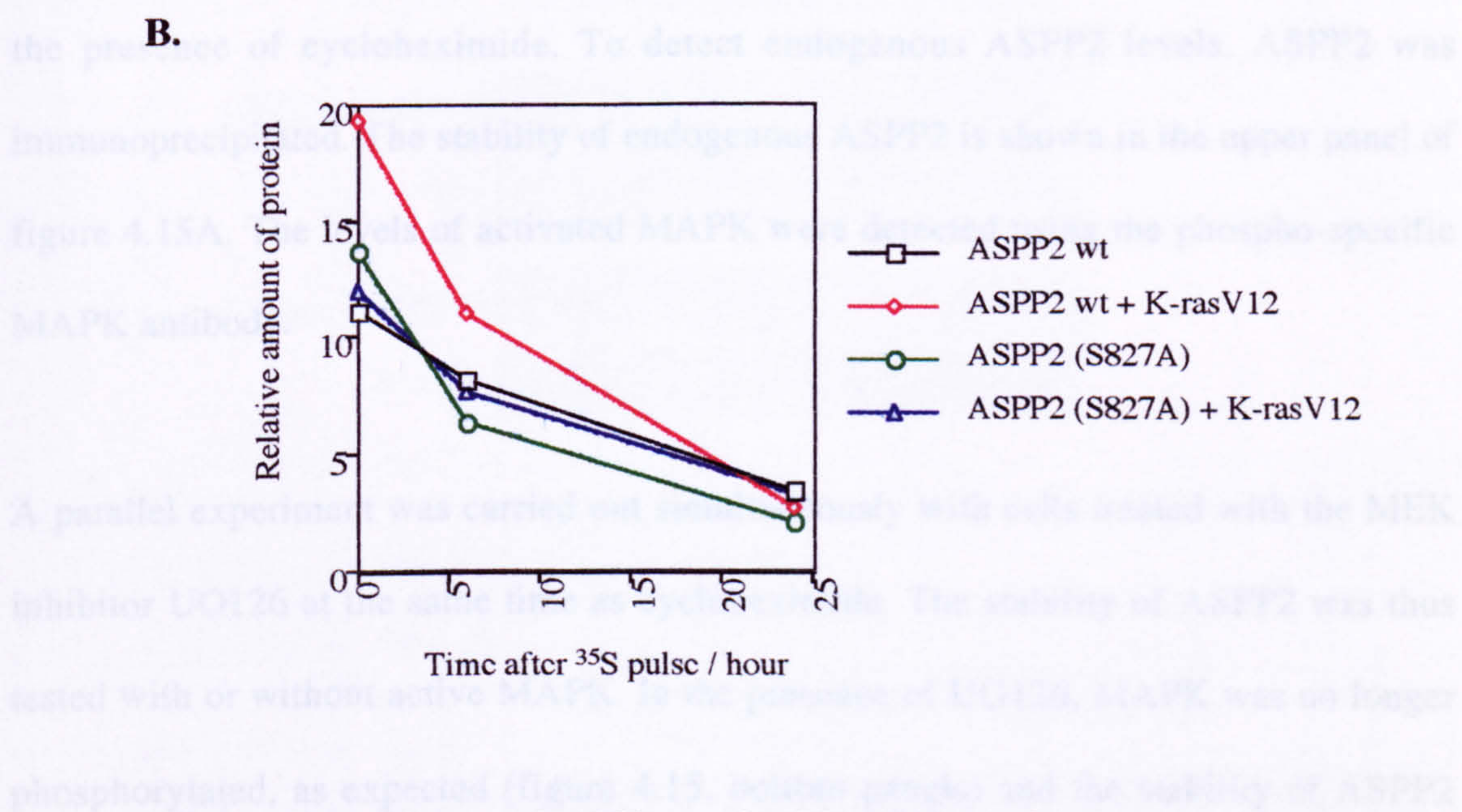
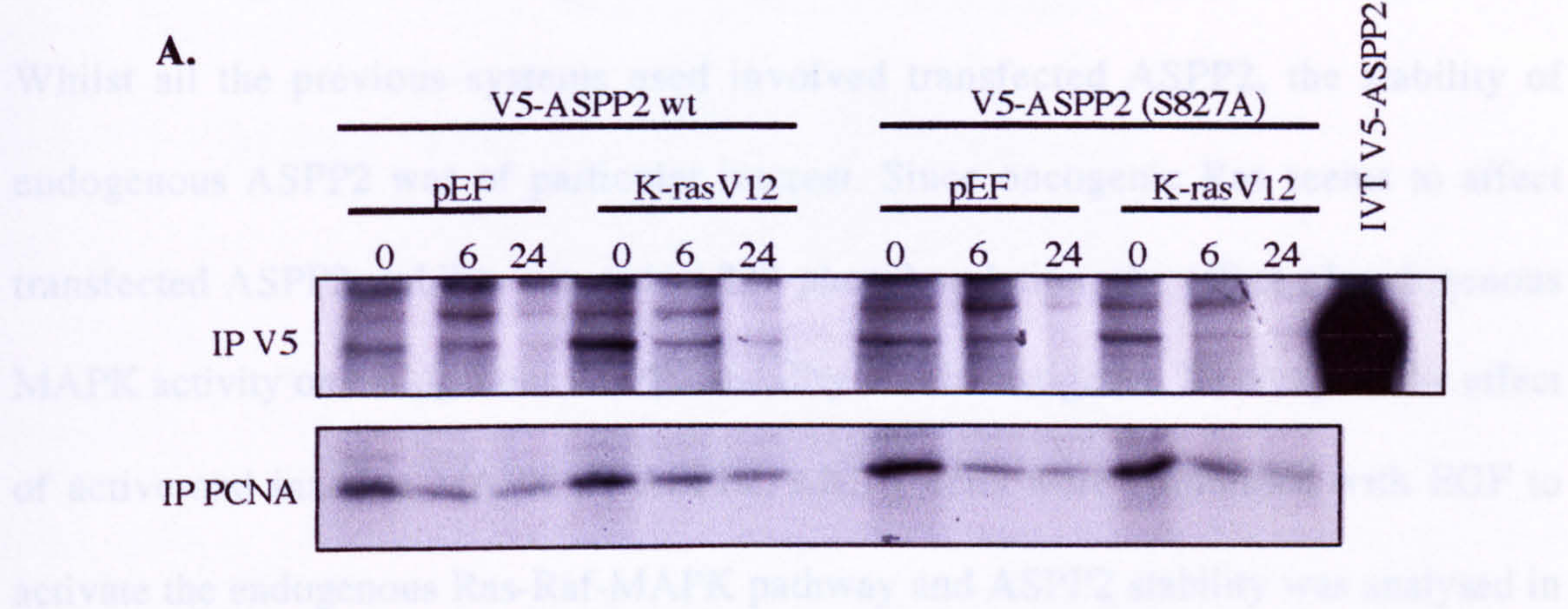


Figure 4.14 Pulse chase of ASPP2 wt and ASPP2 (S827A) in the presence or absence of K-rasV12. (A) Saos2 cells were transfected with ASPP2 wild-type or phosphorylation mutant ASPP2 (S827A) in the presence or absence of K-rasV12. 12 hours after transfection, the cells were pulsed for two hours with 145 μ Ci/ml ³⁵S-labelled methionine and cysteine. The cells were then washed with PBS and unlabelled medium was added to the cells. At the indicated times the cells were lysed and the lysates were first pre-cleared with G-beads then immunoprecipitated with the V5 antibody against transfected ASPP2 for 2 hours. The lysates were then immunoprecipitated with the PC-10 antibody against PCNA as a control. The immunoprecipitates were resolved on SDS-PAGE gels, fixed, amplified and dried before being exposed on a phosphorimager screen to detect the labelled proteins. The upper panel shows the ASPP2 proteins immunoprecipitated with the V5 antibody and the lower panel shows the immunoprecipitated PCNA protein. (B) The intensity of the bands were measured and represented on the graph. The X-axis represents the time after the cells were pulsed with ³⁵S-labelled methionine and cysteine and the Y-axis shows the relative amount of protein.

4.2.8.3. Endogenous ASPP2 requires endogenous MAPK activity for its stability

Whilst all the previous systems used involved transfected ASPP2, the stability of endogenous ASPP2 was of particular interest. Since oncogenic Ras seems to affect transfected ASPP2 stability via serine 827 phosphorylation, the effect of endogenous MAPK activity on endogenous ASPP2 stability was investigated. To compare the effect of active and inactive MAPK on ASPP2, MCF7 cells were stimulated with EGF to activate the endogenous Ras-Raf-MAPK pathway and ASPP2 stability was analysed in the presence of cycloheximide. To detect endogenous ASPP2 levels, ASPP2 was immunoprecipitated. The stability of endogenous ASPP2 is shown in the upper panel of figure 4.15A. The levels of activated MAPK were detected using the phospho-specific MAPK antibody.

A parallel experiment was carried out simultaneously with cells treated with the MEK inhibitor UO126 at the same time as cycloheximide. The stability of ASPP2 was thus tested with or without active MAPK. In the presence of UO126, MAPK was no longer phosphorylated, as expected (figure 4.15, bottom panels) and the stability of ASPP2 was significantly decreased (figure 4.15, upper panels). Quantification of the intensity of the bands clearly showed a difference in ASPP2 stability when MAPK was active compared to when MAPK was inactivated by UO126 (figure 4.15C).

These results indicate that endogenous ASPP2 protein expression can be stabilized by Ras-MAPK activity via the phosphorylation of ASPP2 at serine 827.

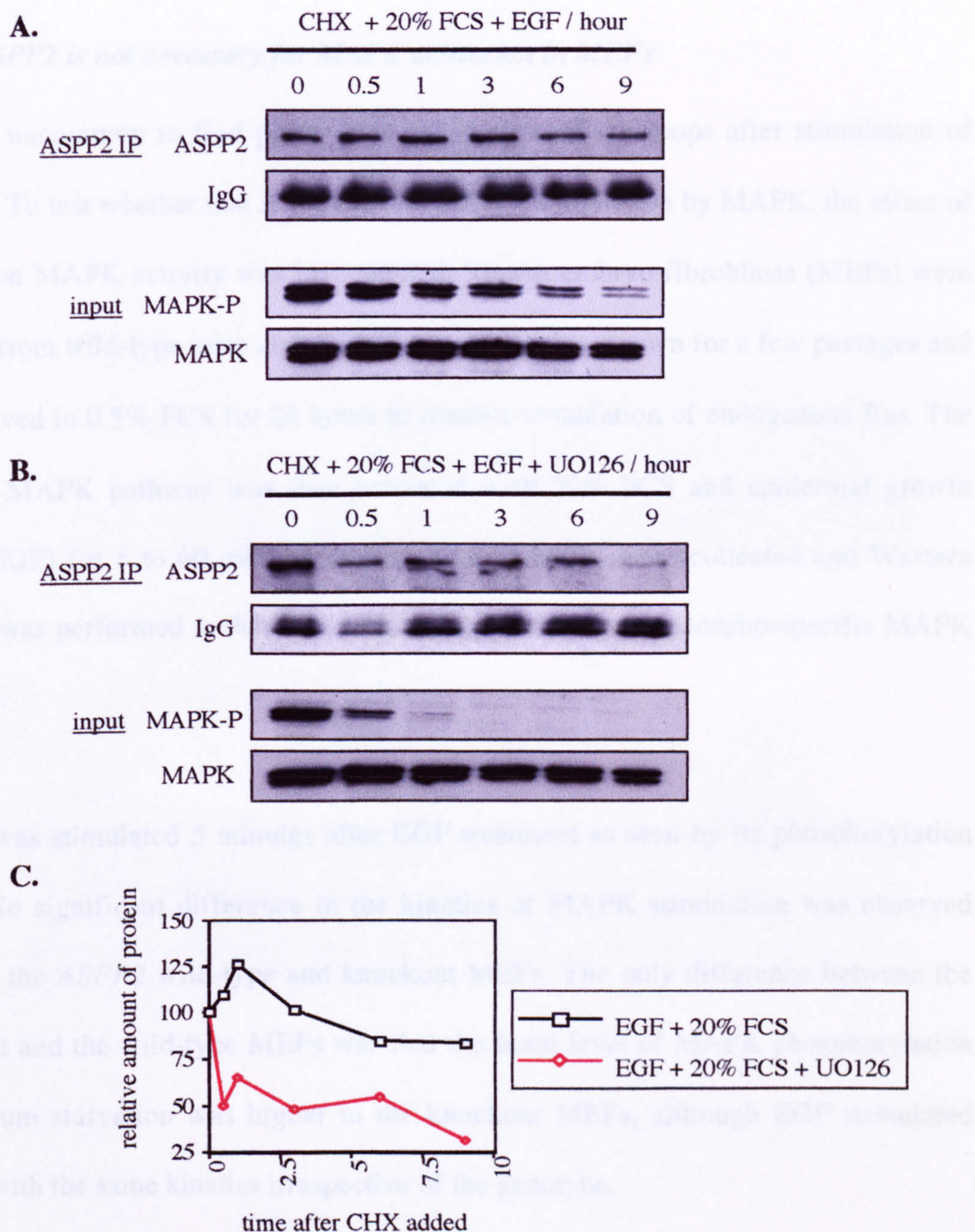


Figure 4.15 *MAPK activity is necessary for endogenous ASPP2 stability.* MCF7 cells were starved of serum for 24 hours in 0.5% FCS. They were then treated with 50 μ g/ml cycloheximide (CHX) and 20% FCS and EGF, without UO126 (**A**) or with UO126 (**B**) for the time indicated. 4mg of lysate was used for immunoprecipitation with the monoclonal 5410 antibody against ASPP2. The immunoprecipitate was western blotted and ASPP2 was detected with the polyclonal bp77 antibody (top panel). The amount of antibody used for the immunoprecipitation was detected with secondary mouse antibody (second panel from the top). 60 μ g of input was western blotted and phosphorylated MAPK was detected with phospho-specific MAPK antibody (third panel from the top) and the total MAPK was detected with a MAPK antibody (bottom panel). (**C**) The intensity of the ASPP2 protein immunoprecipitated was quantified and the bands at 0 hours of CHX addition was set as 100 for both cells.

4.2.9. ASPP2 is not necessary for MAPK activation in MEFs

It is not uncommon to find positive or negative feedback loops after stimulation of proteins. To test whether that is the case for ASPP2 stimulation by MAPK, the effect of ASPP2 on MAPK activity was investigated. Mouse embryo fibroblasts (MEFs) were isolated from wild-type mice and *ASPP2* knockout mice, grown for a few passages and then starved in 0.5% FCS for 24 hours to remove stimulation of endogenous Ras. The Ras-Raf-MAPK pathway was then activated with 20% FCS and epidermal growth factor (EGF) for 5 to 60 minutes (figure 4.16). Lysates were collected and Western blotting was performed to detect MAPK stimulation, using a phospho-specific MAPK antibody.

MAPK was stimulated 5 minutes after EGF treatment as seen by its phosphorylation status. No significant difference in the kinetics of MAPK stimulation was observed between the *ASPP2* wild-type and knockout MEFs. The only difference between the knockout and the wild-type MEFs was that the basal level of MAPK phosphorylation after serum starvation was higher in the knockout MEFs, although EGF stimulated MAPK with the same kinetics irrespective of the genotype.

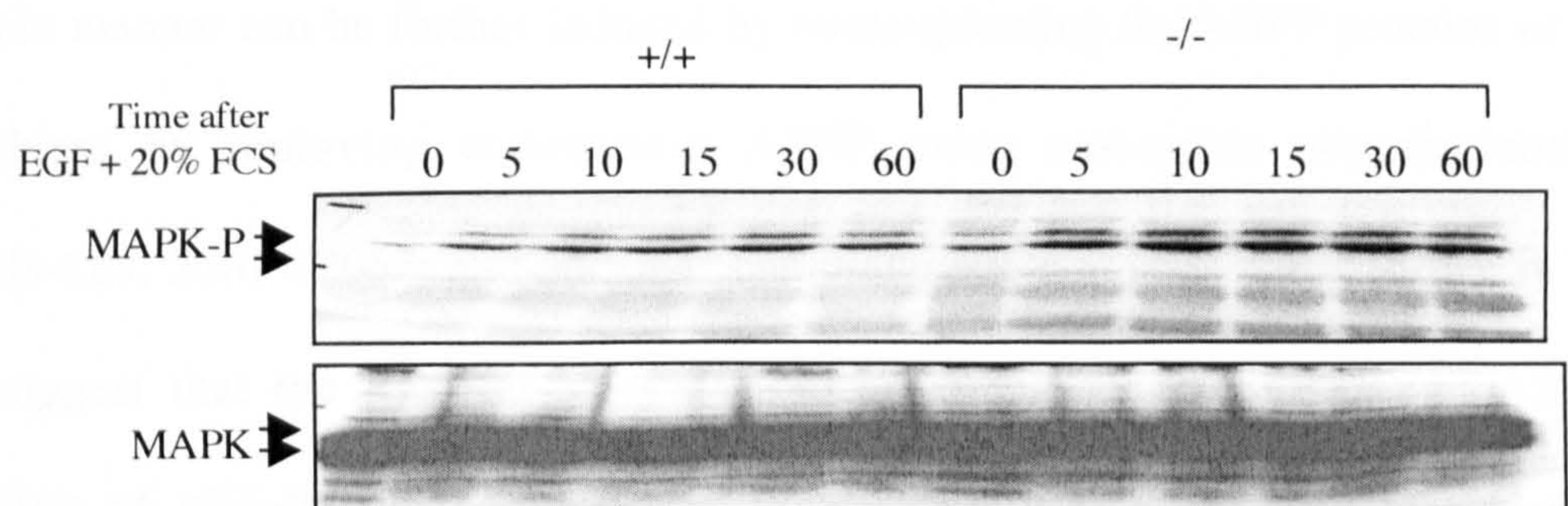


Figure 4.16. *ASPP2 is not necessary for MAPK activation in MEFs.* ASPP2 knockout MEFs and wild-type MEFs were isolated from mice. The cells were starved of serum for 24 hours then treated with EGF and 20% FCS for the times indicated. Lysates were collected and resolved on a Western Blot which was probed with a phospho-specific MAPK antibody (upper panel). The blot was subsequently probed with an antibody against total MAPK (lower panel).

4.2.10. Doxorubicin induces apoptosis partially via the MAPK pathway

ASPP1 and APP2 are known to induce p53-mediated apoptosis in a number of cell lines (Samuels-Lev et al., 2001). Apoptosis induced by chemotherapy drugs in a p53-dependent manner can be further induced by overexpressing the ASPP proteins or can be inhibited by removing endogenous ASPP using anti-sense oligonucleotides {Samuels-Lev, 2001 #2662; and personal communications). The results shown in this thesis suggest that the Ras-Raf-MAPK pathway can mediate ASPP1 and ASPP2 stimulation of p53-dependent apoptosis. It was therefore hypothesised that p53-dependent apoptosis induced by chemotherapy drugs such as doxorubicin might be partially mediated by the Ras-Raf pathway.

To test this hypothesis MCF7 cells, containing wild-type p53, were treated with doxorubicin for 24 hours in the presence or absence of the MEK inhibitor UO126. FACS analysis showed that UO126 had little effect on cells not treated by doxorubicin but could reduce by 30% doxorubicin-induced apoptosis (figure 4.17).

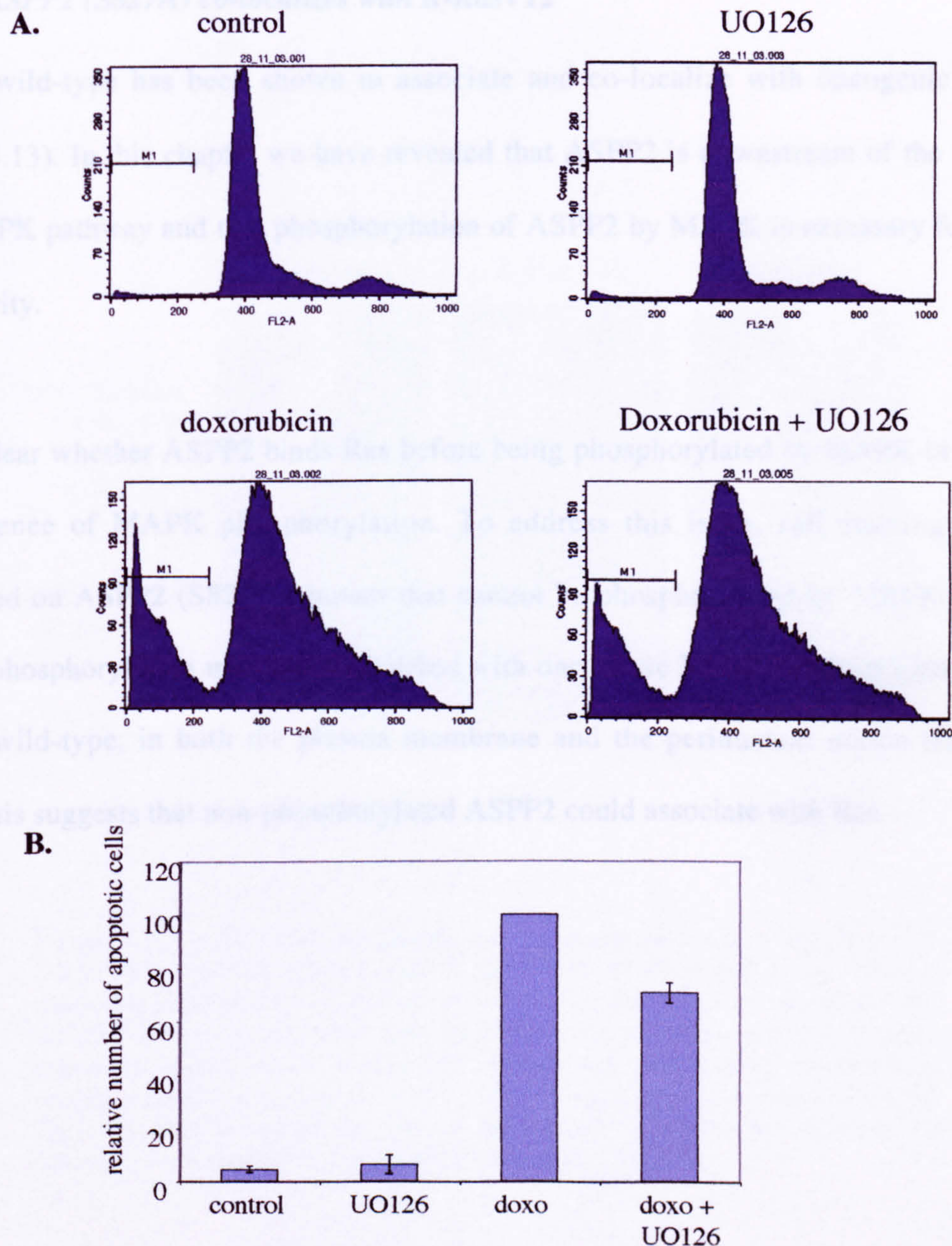


Figure 4.17 MAPK inhibitor UO126 reduces doxorubicin-induced apoptosis in MCF7 cells. MCF7 cells were treated with 4mM doxorubicin (doxo) and 20 μ M UO126 for 24 hours as indicated. The floating and adhering cells were fixed, stained with propidium iodide and analyzed by FACS. (A) Histogram plot showing the cell cycle profile of MCF7 cells. The percentage of apoptotic cells was measured by accumulation of cells with a sub-G1 DNA content (cells in M1 region). (B) Bar graph representing the relative percentage of sub-G1 cells. The mean values were derived from two independent experiments.

4.2.11. ASPP2 (S827A) co-localizes with K-RasV12

ASPP2 wild-type has been shown to associate and co-localize with oncogenic Ras (figure 3.13). In this chapter we have revealed that ASPP2 is downstream of the Ras-Raf-MAPK pathway and that phosphorylation of ASPP2 by MAPK is necessary for its full activity.

It is unclear whether ASPP2 binds Ras before being phosphorylated by MAPK or as a consequence of MAPK phosphorylation. To address this issue, cell staining was performed on ASPP2 (S827A) mutant that cannot be phosphorylated by MAPK. The ASPP2 phosphorylation mutant co-localized with oncogenic Ras to the same extent as ASPP2 wild-type, in both the plasma membrane and the perinuclear region (figure 4.18). This suggests that non-phosphorylated ASPP2 could associate with Ras.

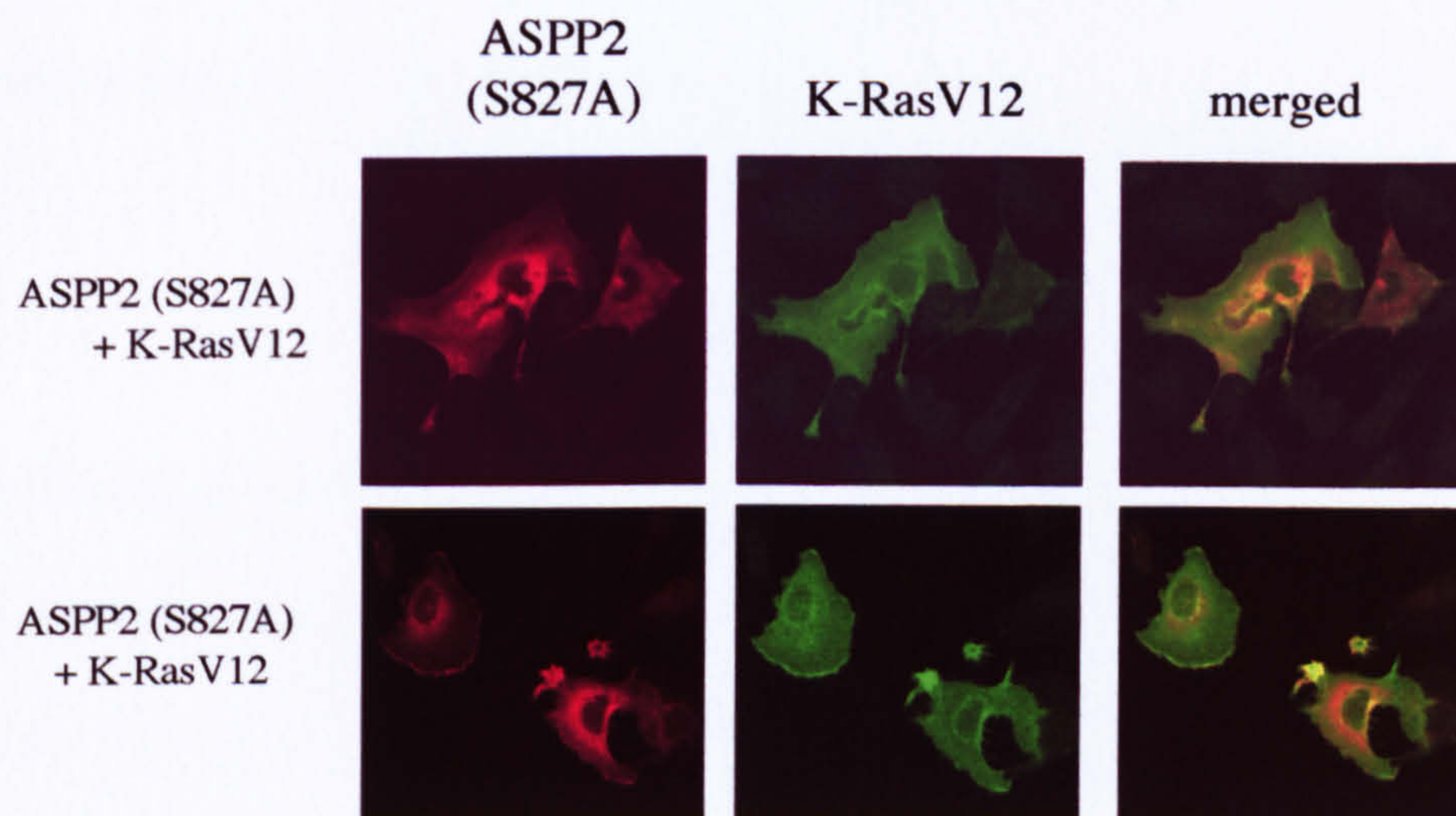


Figure 4.18 *ASPP2 (S827A) phosphorylation mutant co-localizes with K-rasV12.* U2OS cells were transfected with 4 μ g ASPP2 (S827A) and 3 μ g K-rasV12 expression plasmids per 3cm dish. After transfection the cells were starved of serum for 20 hours. The cells were then fixed. The V5 antibody was used to detect transfected ASPP2 mutant and the 259 antibody was used to detect transfected K-rasV12. ASPP2 was stained in red with TRITC and K-rasV12 in green with FITC. The cells were visualized by confocal microscopy. The two rows represent two different sets of staining of mutant ASPP2 and K-rasV12.

4.3 Discussion

In this chapter the pathway involving Ras stimulation of ASPP1 and ASPP2 was investigated. Ras was found to stimulate ASPP1 and ASPP2 activity via the Ras-Raf-MEK-MAPK phosphorylation cascade resulting in MAPK phosphorylating ASPP1 and ASPP2. Phosphorylation of ASPP2 by MAPK resulted in increased stability of ASPP2 protein.

4.3.1. Ras stimulates ASPP2 via the Raf pathway

To investigate whether Ras could stimulate ASPP1 and ASPP2 directly or whether this stimulation was via the Raf downstream effector pathway, an active form of Raf was used. Raf-CAAX contains the membrane localization signal of K-ras4B and is constitutively active, not requiring Ras stimulation for its activity {Leevers, 1994 #2798}. In this chapter Raf-CAAX has been shown to stimulate p53 pro-apoptotic transactivation activity via ASPP2. The fact that ASPP2 is stimulated by Raf-CAAX to the same extent as it is by oncogenic H-RasV12 and K-RasV12 is strongly supportive of ASPP2 being stimulated by RasV12 via its downstream effector Raf CAAX.

4.3.2. ASPP2 is phosphorylated by MAPK in vitro and in vivo

Raf is upstream of a phosphorylation cascade that involves Raf activation of MEK by phosphorylation (Ahn et al., 1991; Cowley et al., 1994; Dent et al., 1992; Gomez and Cohen, 1991; Huang et al., 1993; Kosako et al., 1992; Kyriakis et al., 1992; Nakielnny et al., 1992), followed by MEK activation of MAPK by tyrosine and threonine phosphorylation (Crews et al., 1992; Kosako et al., 1992; Matsuda et al., 1992). MAPK is a serine/threonine kinase and is known to phosphorylate a number of downstream

effectors such as Rsk, p62TCF, NF-IL6, c-myc, Elk-1 and Ets-2 (Chen et al., 1992; Gille et al., 1992; Marais et al., 1993; Marshall, 1995; Nakajima et al., 1993; Seth et al., 1992). Sequence alignments and point mutation analysis of MAPK substrates lead to the discovery of the MAPK consensus phosphorylation site (Alvarez et al., 1991; Gonzalez et al., 1991). Analysis of ASPP1 and ASPP2 protein sequence pointed to two putative MAPK phosphorylation sites in each of the two proteins. Both these putative phosphorylation sites were present in regions of high homology between ASPP1 and ASPP2. However, following an *in vitro* phosphorylation assay, only the second putative MAPK phosphorylation site was shown to be phosphorylated by recombinant MAPK1.

Phosphorylation on serine 827 of ASPP2 by MAPK was confirmed using a phospho-specific antibody against ASPP2: endogenous Ras stimulation by EGF resulted in MAPK activation, followed shortly by ASPP2 phosphorylation *in vivo*. Since the antibody specifically recognizes the phosphorylated serine 827 of ASPP2 and that is the site shown to be phosphorylated by MAPK *in vitro*, we concluded that ASPP2 was phosphorylated on serine 827 *in vivo* by MAPK. Thus, ASPP2 is a novel MAPK effector.

Active MAPK can phosphorylate its substrates both in the cytoplasm, such as Rsk, or in the nucleus, such as Ets-2 and Elk-1. As ASPP2 is also present in both the cytoplasm and the nucleus, MAPK could theoretically phosphorylate ASPP2 in either of those cellular compartments. It would be interesting to see by immunofluorescence whether these two proteins co-localize and if so, where. As the association of the two proteins is likely to be transient, a time course after MAPK stimulation would be necessary for this experiment.

4.3.3. MAPK activity is necessary for full ASPP1 and ASPP2 activity

Since ASPP2 was shown to be phosphorylated *in vivo* by MAPK, the functional significance of this signal was investigated. To do so, UO126 and PD98059, known MAPK inhibitors, were used (Favata et al., 1998). Blocking endogenous MAPK by UO126 or PD98059 showed a marked reduction in ASPP2 activity, similar to that seen when endogenous Ras was inhibited by RNAi (chapter 3). This would suggest that endogenous Ras stimulation of ASPP2 is via MAPK activation. ASPP2 phosphorylation mutants confirmed that it was indeed phosphorylation of serine 827 by MAPK that mediates ASPP2 stimulation by Raf-CAAX.

Since ASPP2 phosphorylation at serine 827 is required for Ras-Raf-MAPK stimulation, it was surprising that the ASPP2 (S827A) was able to transactivate p53 to the same extent as ASPP2 wild-type and the control mutant. However, this could be explained by the conditions used to perform the experiment: in order to see the effect of Raf-CAAX on ASPP2 activity, the cells were grown in 0.5% FCS to remove background stimulation of Ras. Therefore, in those conditions, there is low endogenous Ras activity and as a consequence low amounts of phosphorylated ASPP2. Thus, in the absence of Ras stimulation, ASPP2 (S827A) has a similar activity to ASPP2 wild-type. Only when MAPK is activated and phosphorylates ASPP2 can a difference be seen between the activity of the ASPP2 phosphorylation mutant at serine 827 that cannot be phosphorylated and that of the wild-type, phosphorylated ASPP2. Indeed, in conditions where endogenous Ras was stimulated by 10% FCS, a difference was seen in the activity of ASPP2 (S827A) compared to ASPP2 wild-type: only ASPP2 wild-type was able to synergize with p53 to induce apoptosis (figure 4.10).

The MAPK phosphorylation site of ASPP2 is homologous to a putative MAPK phosphorylation site in ASPP1. Mutagenesis was performed on ASPP1 to generate two phosphorylation mutants. A transactivation assay showed that ASPP1 wild-type and ASPP1 (S671A) responded to K-RasV12 stimulation but ASPP1 (S746A) was unable to be stimulated by K-RasV12. Thus, the ASPP1 and ASPP2 MAPK phosphorylation site is conserved and MAPK phosphorylation at serine residues 746 and 827, respectively, results in increased pro-apoptotic activity.

The model discussed in Samuels-Lev et al. suggests that ASPP2 interacts with p53 to increase its binding to pro-apoptotic reporters (Samuels-Lev et al., 2001). In order for this to happen, ASPP2 is expected to be present in the nucleus with active p53. However ASPP2 is predominantly found in the cytoplasm with some minimal presence in the nucleus and little evidence has been found so far about its cytoplasmic-nuclear shuttling. One model could be that the Ras-Raf-MAPK stimulation of ASPP2 would lead to its shuttling into the nucleus after phosphorylation on serine 827 as has been shown to be the case for other downstream Ras-Raf-MAPK signalling effectors such as Rsk (Chen et al., 1992). Up to date, however, no difference in ASPP2 localization has been observed in the presence of oncogenic Ras. Nonetheless, one would expect that ASPP2 presence in the nucleus could be a transient process as it would eventually lead to apoptosis by p53-dependent transactivation of pro-apoptotic genes and would therefore have to be tightly regulated. One possibility could be that ASPP2 activity is regulated by nuclear export, such as is the case with p53 (Roth et al., 1998). Therefore to observe the presence of ASPP2 in the nucleus after Ras-MAPK activation, ASPP2 nuclear export should be inhibited, such as by using for example the CRM-1-mediated nuclear export inhibitor, leptomycin B. Figure 4.5 shows that ASPP2 is phosphorylated

one hour after EGF stimulation in Saos2 cells so a time course based around that time point could be used to test ASPP2 shuttling to the nucleus.

Although no difference has so far been seen in ASPP2 cellular localization following oncogenic Ras stimulation, the same has not been true for ASPP1. As mentioned in chapter 3, K-RasV12 co-expression results in a significant change in ASPP1 localization, forming large, brightly stained, doughnut-like shapes. The ASPP1-containing globules are present in the cytoplasm and often seen in the perinuclear region. As ASPP1 is known to be stimulated by Ras via the Raf-MEK-MAPK pathway, we investigated whether the change in ASPP1 immunofluorescence was due to the MAPK pathway. Addition of UO126, the MAPK inhibitor, showed that the change in ASPP1 cellular localization following oncogenic Ras stimulation was indeed caused by endogenous MAPK activity. Although Ras can stimulate ASPP1 and ASPP2 pro-apoptotic activity to the same extent, and does so via the Raf-MEK-MAPK phosphorylation cascade resulting in MAPK phosphorylation of both proteins at a homologous serine, there seems to be a difference in the extent of ASPP1 activation compared to ASPP2 as seen by cell staining.

To investigate whether endogenous Ras could lead to a change in cellular localization of endogenous ASPP1, normal human fibroblasts (NHF) were used. ASPP1 levels are very low in tumour cell lines and endogenous protein levels cannot be visualized by cell staining in those cells. Primary cells such as NHF, however, have higher levels of endogenous ASPP1 and ASPP2. This suggests that tumour cells selectively reduced ASPP1 and ASPP2 expression levels. Since ASPP proteins are tumour suppressor proteins, a reduced expression of ASPP would allow tumour cells clonal selectivity.

NHF cells also have wild-type Ras expression. Thus NHF cells were used to test the effect of endogenous Ras on ASPP1 staining. Stimulation of NHF with EGF did not show any change in ASPP1 localization. For the reasons mentioned above, this experiment should be repeated in the presence of the nuclear export inhibitor, leptomycin B, to counteract any possible shuttling that might prevent visualization of ASPP1 nuclear transportation after Ras stimulation. Alternatively, subcellular fractionation could be used to detect an increased presence of ASPP in the nucleus following Ras stimulation.

4.3.4. MAPK phosphorylation of ASPP2 increases its stability

When ASPP1 or ASPP2 were co-transfected with either H-RasV12 or K-RasV12, Western blotting consistently showed that ASPP expression levels were slightly increased compared to samples that were not co-transfected with oncogenic Ras. However, the mutant ASPP1 (S746A) that couldn't be stabilised by K-RasV12 did not have its expression levels increased in the presence of K-RasV12 (figure 4.9C). This suggested that oncogenic Ras might increase ASPP1 and ASPP2 activity by affecting the post-translational modification pattern of these proteins. This trend was confirmed when the UO126 MAPK inhibitor was used to reduce ASPP2 activity: Western blotting showed that transfected ASPP2 levels were markedly decreased in the presence of UO126 compared to control samples. Thus, we hypothesised that Ras could stimulate ASPP activity by affecting its expression levels and that this effect was mediated by the Raf-MEK-MAPK signalling pathway. Therefore we suggested ASPP1 and ASPP2 phosphorylation by MAPK on serines 746 and 827, respectively, could lead to increased ASPP stability.

To test this hypothesis ASPP2 was transfected in the presence or absence of H-RasV12 and its stability measured after the addition of the translation inhibitor, cycloheximide. ASPP2 levels were seen to be increased by H-RasV12. In samples without H-RasV12 co-transfection, ASPP2 half-life seemed to be around 6 hours whereas in samples co-transfected with H-RasV12 the ASPP2 levels at 6 hours had not diminished. Confirming that the induction of ASPP2 stability by Ras was via ASPP2 phosphorylation, oncogenic Ras was unable to increase protein levels of the ASPP2 (S827A) phosphorylation mutant. It could therefore be concluded that oncogenic Ras increased ASPP2 protein levels by phosphorylation.

There are two possible mechanisms that could explain ASPP2 increased expression by Ras. One could be via an increased rate of ASPP2 translation and another could be by increasing ASPP2 protein stability. Literature has given many examples of protein phosphorylation leading to increased protein stability whereas little is known about translation regulation, especially caused by change in phosphorylation status. In order to differentiate between the two possible mechanisms that Ras could use to increase ASPP2 expression levels, a pulse-chase experiment was done. Cells were pulsed with ³⁵S-labelled methionine and cysteine for two hours and chased with normal non-radioactive medium for 0, 6 or 24 hours. This method allowed us to differentiate between the Ras effect on ASPP2 being caused by an increase in translation or an increase in ASPP2 stability: protein translation should not be affected by pulsing the cells but protein stability can be monitored. Unfortunately the endogenous levels of ASPP2 were too low to see any labelled protein after immunoprecipitation. Therefore we transfected ASPP2 in Saos2 cells in the presence or absence of K-RasV12 and looked at the levels of transfected ASPP2 over time.

The results shown in figure 4.13 are difficult to decipher. On one hand there was a significant increase in ASPP2 protein expression after K-RasV12 co-transfection at time 0, which was not seen with the phosphorylation mutant ASPP2 (S827A). On the other hand, 6 hours after chasing, the ASPP2 levels were identical in cells co-transfected with K-RasV12 compared to those transfected alone. There are different possible interpretations to these results. One interpretation is that K-RasV12 can decrease ASPP2 protein stability as the rate degradation was steeper when ASPP2 was co-transfected with Ras. Another interpretation could be that K-RasV12 increased ASPP2 stability. The cells were pulsed for two hours which would be a sufficient amount of time to show a significant difference in protein levels of ASPP2 if K-RasV12 increased its stability, as seen at time 0. It would then be expected that ASPP2 would be negatively regulated by another mechanism in the long term which would explain its decreased expression levels over time. If this second theory is correct, then we would expect that a shorter pulse followed by shorter time points would show ASPP2 levels at time 0 to be equivalent in the presence and absence of Ras, and ASPP2 to be stabilized in a short time course when co-transfected with K-RasV12 compared to ASPP2 alone. Although the experiment with a shorter pulse and shorter time points was attempted, due to technical problems experienced no conclusion could be made.

Whether K-RasV12 leads to increased ASPP2 stability or to decreased ASPP2 stability, what could be concluded is that its effect on ASPP2 is mediated by ASPP2 phosphorylation on serine 827 because the effect of K-RasV12 is abrogated in the phosphorylation mutant.

To address the issue of Ras stabilization of ASPP2 further, an experiment was done looking at endogenous ASPP2 and endogenous Ras. Since the effect of Ras stability on ASPP2 was shown to be mediated by MAPK phosphorylation, the MAPK inhibitor UO126 was used. MCF7 cells were starved and then stimulated with EGF in the presence of cycloheximide. In parallel, UO126 was added to an identical sample so see the effect of MAPK on ASPP2 stability. Since endogenous ASPP2 levels are low in cells, an immunoprecipitation was performed to detect the ASPP2 signal. The stability of endogenous ASPP2 was clearly shown to be decreased in the presence of UO126. This proves that endogenous MAPK activity is necessary for ASPP2 stability.

Although there have been some contradictions in the results presented so far, the overwhelming majority of results suggest that ASPP2 is stabilized in the presence of Ras, via the Raf-MEK-MAPK pathway. The controversy stems from the pulse chase result, which was inconclusive, and the fact that Ras RNAi was shown, in one experiment, to lead to increased ASPP1 (but not ASPP2) expression levels (figure 3.20). This effect of Ras RNAi on ASPP1 was only seen when the cells were treated with cisplatin but not when the cells were left untreated. However, all other results indicated that endogenous and oncogenic Ras lead to increased ASPP stability: co-transfection of oncogenic Ras with ASPP1 and ASPP2 invariably lead to increased ASPP protein levels; the MAPK inhibitor UO126 lead to reduced ASPP2 levels; and cycloheximide experiments with transfected and endogenous ASPP2 showed an increase in stability caused by oncogenic and endogenous Ras activity. Taken together, we can conclude that Ras does indeed lead to increased ASPP1 and ASPP2 stability via their phosphorylation by MAPK.

4.3.5. ASPP2 is not necessary for MAPK stimulation in MEFs

Stimulation of proteins is often a tightly controlled process. It is not uncommon to have either positive or negative feedback loops to allow the necessary response needed for a particular stimulus. Stimulation of ASPP2 by the Ras-Raf-MEK-MAPK pathway has been demonstrated in this chapter. To assess whether ASPP2 could itself have an impact on the MAPK activity, resulting in a feedback mechanism, *ASPP2* knockout MEFs were used. Stimulation of endogenous MAPK by EGF and FCS showed no difference in the kinetics of MAPK phosphorylation between wild-type MEFs and *ASPP2*-null MEFs, suggesting that ASPP2 is downstream only, and not upstream of MAPK. However, MAPK showed higher basal levels of phosphorylation in *ASPP2*-knockout cells compared to those with *ASPP2* wild type. This suggests that ASPP2 might actually inhibit MAPK phosphorylation at basal levels, and therefore might act in a negative feedback loop with MAPK.

Studies with *ASPP2* knockout mice showed a very strong phenotype, particularly in the brain, which is found to be significantly enlarged compared to wild-type mice (Virginie Vives, unpublished data). Thymocytes from these knockout mice also showed a difference in apoptotic activity compared to thymocytes from wild-type mice, suggesting ASPP2 plays a crucial role in regulating apoptosis in those cell lines (Virginie Vives, unpublished data). However, no difference in phenotypes was observed in MEFs when comparing *ASPP2* knockout and wild-type. This suggests that ASPP2 does not play a major role in MEFs. In order to fully investigate the effect that ASPP2 could have on MAPK, a cell line should be used where ASPP2 is known to have a physiological effect. Therefore, to fully evaluate whether ASPP2 has an effect on MAPK activity, a similar experiment to that shown in this chapter should be performed

in thymocytes or brain tissue, comparing MAPK stimulation in ASPP2 knockout and wild-type cells.

4.3.6. Endogenous MAPK activity is necessary for full apoptotic response to doxorubicin

ASPP1 and ASPP2 are known to increase apoptosis in response to chemotherapy drugs (Samuels-Lev et al., 2001). Since MAPK has been shown to be upstream of ASPP1 and ASPP2, the effect of MAPK activity on doxorubicin-induced apoptosis was investigated. Doxorubicin is a strong inducer of apoptosis and ASPP1 and ASPP2 are known to be involved in its apoptotic pathway. This has been shown using anti-sense ASPP1 and ASPP2 which significantly reduced doxorubicin-induced apoptosis, as measured by flow cytometry (Dr Bergamaschi, personal communication). To test whether MAPK is involved in doxorubicin-mediated apoptotic signalling, doxorubicin was used to treat MCF7 cells, in the presence or absence of UO126, a MAPK inhibitor. UO126 was shown to reduce by 20% doxorubicin-mediated apoptosis. This suggests that MAPK activity is necessary for a full doxorubicin-induced apoptotic response. Previous work however, has shown that the Ras-Raf-MAPK pathway could inhibit doxorubicin-induced apoptosis (Kwok et al., 1994; Nooter et al., 1995).

The Ras-Raf-MEK-MAPK pathway is known to induce a number of different responses, such as proliferation, differentiation and apoptosis. Its actual response depends on a number of different factors such as duration of extracellular signalling and cell type. Another factor that might affect the response after Ras-Raf stimulation might be the expression level of the downstream targets of MAPK. ASPP1 and ASPP2 are known to be at reasonably high levels in MCF7 cells, it is therefore not unexpected that

MAPK activation will stimulate the ASPP to induce apoptosis. In cells with low ASPP expression, activation of ASPP by MAPK might be insignificant and the Ras-Raf-MAPK response might therefore not lead to apoptosis but to proliferation or differentiation. Thus the expression levels of ASPP1 and ASPP2 might be another factor to take into account when predicting the response induced by the Ras pathway.

4.3.7. ASPP2 (S827A) co-localizes with K-RasV12

So far, there seems to be two events linking ASPP2 and Ras. Firstly, ASPP2 can bind activated Ras and secondly, ASPP2 is stimulated by the downstream effector of Ras, MAPK. It is unclear whether ASPP2 binds Ras prior to its phosphorylation by MAPK or as a consequence of its phosphorylation. To address this issue, cell staining was performed with the ASPP2 (S827A) mutant that cannot be phosphorylated by MAPK. ASPP2 (S827A) was seen to co-localize with Ras to the same extent as wild-type ASPP2, in both the plasma membrane of U2OS cells and in the perinuclear region. This strongly suggests that ASPP2 phosphorylation by MAPK is not necessary for its binding to Ras. Whether ASPP2 binding to Ras is necessary for its stimulation by MAPK remains to be investigated.

4.4. Scope of study

In this chapter we have dissected the mechanism for Ras stimulation of ASPP1 and ASPP2. ASPP1 and ASPP2 are stimulated by the Ras-Raf-MEK-MAPK signalling pathway. ASPP2 has been shown to be a substrate to the MAPK kinase both *in vitro* and *in vivo*. MAPK phosphorylation is necessary for full ASPP activity and it has been shown to phosphorylate ASPP1 and ASPP2 on their serines 746 and 827, respectively. Phosphorylation of ASPP2 by MAPK leads to increased stability of ASPP2. The increased protein stability of ASPP2 following Ras-Raf-MAPK stimulation could explain the results in the previous chapter where Ras was seen to increase ASPP1 and ASPP2 activity. Thus, a novel downstream substrate of the Raf-MAPK phosphorylation cascade has been discovered.

Chapter 5

Regulation of ASPP1 and ASPP2 activity

5.1 Introduction

ASPP1 and ASPP2 proteins increase p53 pro-apoptotic activity (Samuels-Lev et al., 2001). Since the presence of functional ASPP proteins could lead to an increase in apoptosis, it is likely that the activity of these proteins will be tightly regulated. In this chapter I examine potential mechanisms by which ASPP1 and ASPP2 activity can be regulated.

In chapter 3, ASPP1 and ASPP2 are shown to contain the Ras-association domain in their amino terminus. This region is necessary for full ASPP function. The first part of this chapter will describe how deletion mutants were used to investigate in more detail what role these amino-termini have in ASPP function.

Post-translational modification is a common mechanism by which protein function is regulated. The most well-studied modifications are phosphorylation, acetylation and ubiquitination. These modifications allow rapid induction or inhibition of a protein's activity and are used for signals to be transduced from one protein to another resulting in a rapid cascade and amplification of signalling. Post-translational modifications are particularly common in proteins regulating cell cycle and in those sensing stress signals. p53 is known to be regulated by a multitude of modifications, including phosphorylation, acetylation, ubiquitination and sumoylation (Brooks and Gu, 2003; Melchior and Hengst, 2002; Xu, 2003). p53 is an unstable and inactive protein in normal cycling cells. However after DNA damage or other stress signals it is stabilized

and activated by an array of modification. Phosphorylation of p53, which occurs on a number of residues in its amino and carboxy terminus, has been shown to stabilize the protein and increase its activation, as does acetylation in the carboxy-terminus of p53 (Brooks and Gu, 2003; Xu, 2003). Ubiquitination, mediated by the p53 negative regulator Mdm2, however, results in p53 export from the nucleus and increased degradation (Haupt et al., 1997; Kubbutat et al., 1997; Kubbutat et al., 1998; Roth et al., 1998). As expected, p53 post-translational modifications are tightly controlled as they can lead to either cell cycle arrest, apoptosis or allow the cell to continue cycling.

Not only is p53 function affected by post-translational modifications, but other proteins that regulate p53 are also subject to post-translational modifications that can affect their function. For example, Mdm2, the most well-studied p53 negative regulator, is subject to phosphorylation. As mentioned in section 1.4.5, when unmodified, Mdm2 can inhibit p53 activity by exporting p53 to the cytoplasm and directing its degradation by ubiquitination. Mdm2 has been shown to be phosphorylated by a number of kinases such as DNA-PK, ATM, Akt, p38 SAPK and Cdk (Alarcon-Vargas and Ronai, 2002; Khosravi et al., 1999; Maya et al., 2001; Mayo and Donner, 2001; Mayo et al., 1997a; Zhang and Prives, 2001). Phosphorylation of Mdm2 by these kinases results in reduction of its stability and therefore leads to increased p53 stability and activity, with an overall increase in p53-dependent cell cycle arrest or apoptosis.

ASPP1 and ASPP2 are known positive regulators of p53 and can specifically promote p53-dependent transactivation of pro-apoptotic genes. As with all elements of the p53-signalling pathway, their function is thought to be tightly regulated. It was therefore hypothesised that ASPP function, like that of p53 and Mdm2, might be regulated by

post-translational modifications, such as phosphorylation. I have shown in chapter 4 that ASPP2 can indeed be phosphorylated by MAPK1 and that this phosphorylation increases its activity by stabilizing the protein. In the second part of this chapter, I examine other putative ASPP2 kinases and whether they can affect ASPP2 function.

5.2. Results

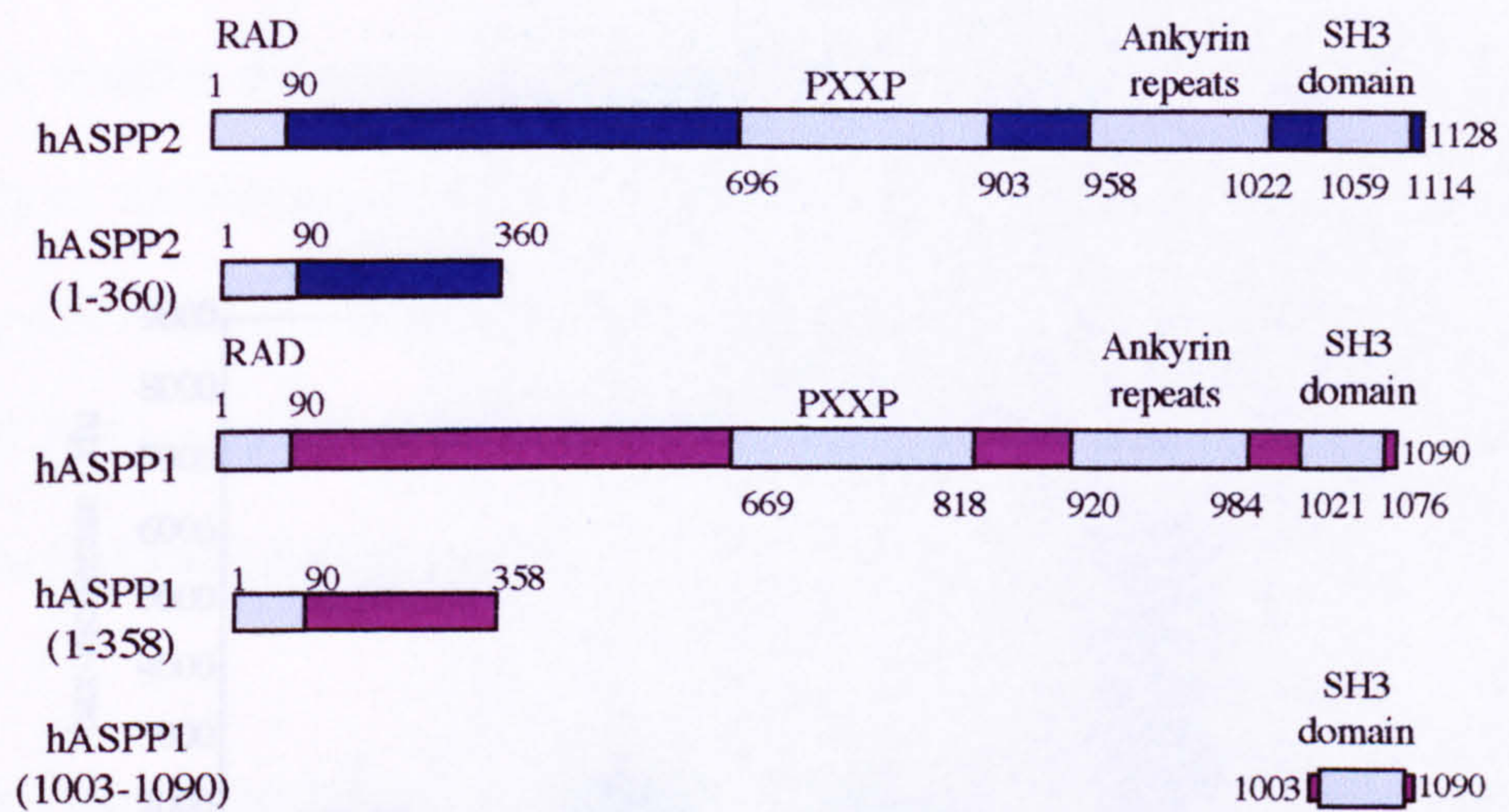
5.2.1. The amino terminus of ASPP2 stimulates p53 activity

In chapter 3 it was shown that the amino terminus of ASPP1 binds Ras. It is unclear whether ASPP binding to Ras occurs before or after ASPP stimulation by Ras through the Ras-Raf-MAPK pathway. Cell staining data suggested that phosphorylation by MAPK was not necessary for ASPP2 to associate with oncogenic Ras (figure 4.18).

To analyze this further, ASPP1 and ASPP2 deletion constructs expressing only the amino termini were used (figure 5.1). These fragments were expected to compete with full-length ASPP to bind to Ras, and therefore act as dominant negatives. I hypothesised that, if ASPP binding to Ras is necessary for its stimulation by the Ras-MAPK pathway, the presence of these amino terminal fragments would inhibit MAPK activation of ASPP1 and ASPP2. If, however, ASPP binding to Ras has no direct consequence on MAPK stimulation of ASPP, then the presence of these amino terminal fragments would have no effect on ASPP-dependent stimulation of p53.

To test which scenario occurred in cells, Saos2 cells were transfected with p53 expression plasmids in the presence or absence of ASPP2 (1-360) expression plasmids. This fragment was expected to compete with endogenous ASPP2 to bind to endogenous Ras. Unexpectedly, p53 transactivation activity was shown to be increased by the ASPP2 (1-360) fragment (figure 5.2). This result was reproducible and seen in several independent experiments.

HUMAN



MOUSE

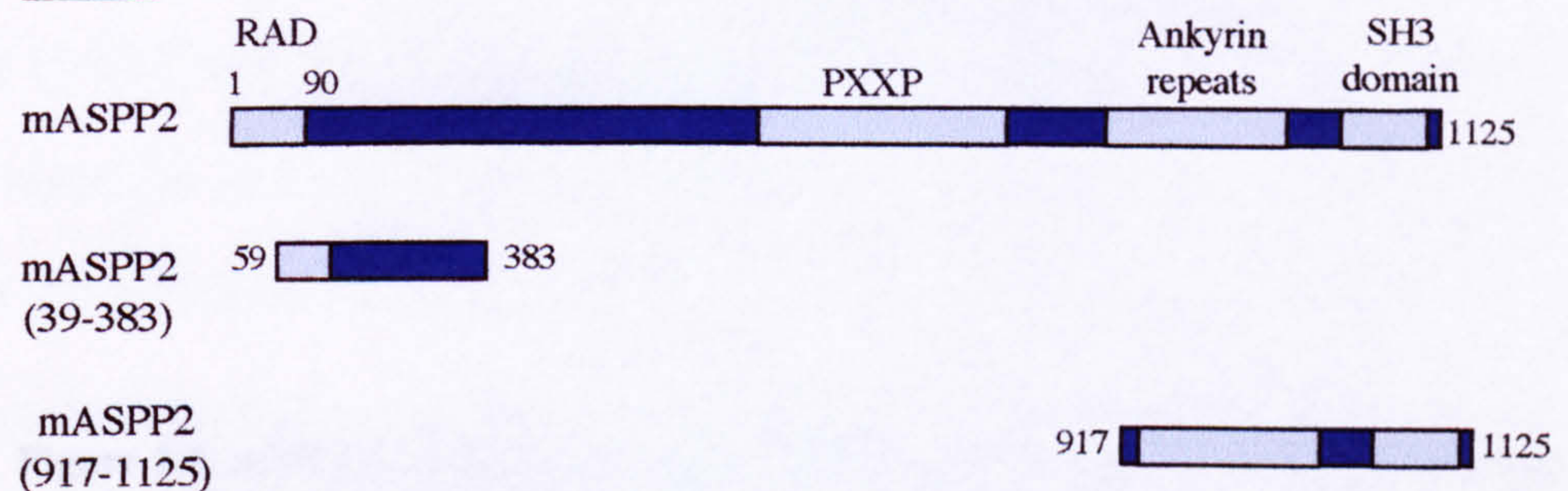


Figure 5.1. Schematic diagram of mouse and human ASPP1 and ASPP2 deletion fragments. Deletion mutants were made of ASPP1 and ASPP2 as indicated in mammalian expressing pCDNA3 plasmids. The prefix “h” stands for human and “m” for mouse. Construction of these plasmids was done by Dr Susana Llanos and Alan Renton, as described in Materials & Methods.

5.2.2. The amino terminus of ASPP1 or ASPP2 stimulates p53 activity via full-length ASPP1 and ASPP2

To test whether the effect of the amino terminal fragments of ASPP stimulated p53 directly or via full-length ASPP1 and ASPP2, these fragments were co-transfected full-

length ASPP1 (Fig. 5.2). Co-transfection of ASPP1 (1-358) increased p53 and ASPP1 synergistically. Similarly, co-transfection of ASPP2 (1-360) increased p53 and ASPP2 synergistically.

In this experiment, 50 ng of pCDNA wild type p53 was transfected into Saos2 cells in the presence or absence of 2 μ g ASPP2 (1-360). 1 μ g Bax-luciferase was transfected per 6 cm dish. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. The mean values were derived from two independent experiments.

ASPP1 (1-358) and ASPP2 (1-360) stimulation of p53 is dependent on full-length ASPP since the deletion fragments alone did not stimulate p53 to the same extent. ASPP1 (1-358) even inhibited p53 activity in the absence of full-length ASPP1, although this experiment was only performed once and needs to be repeated.

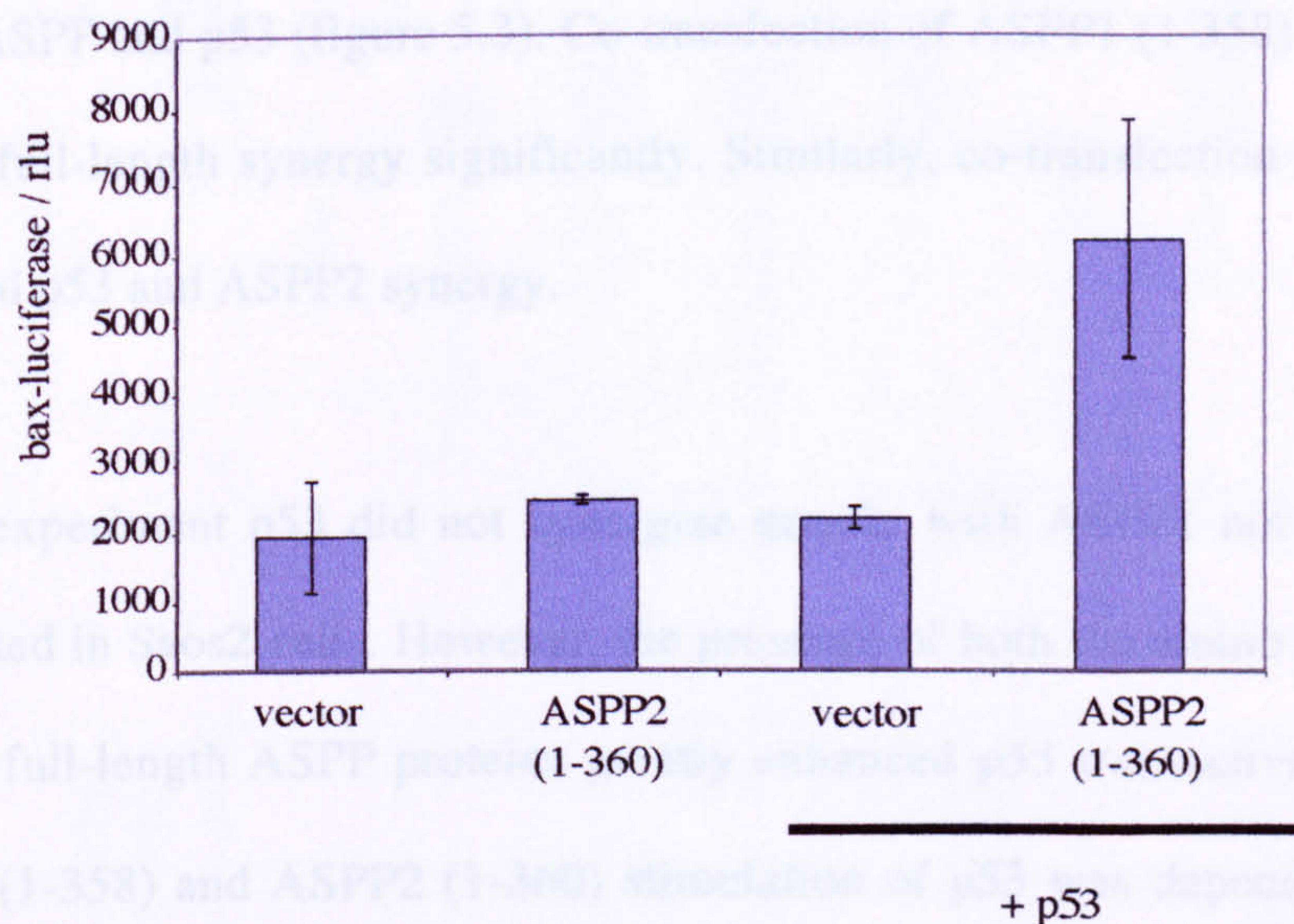


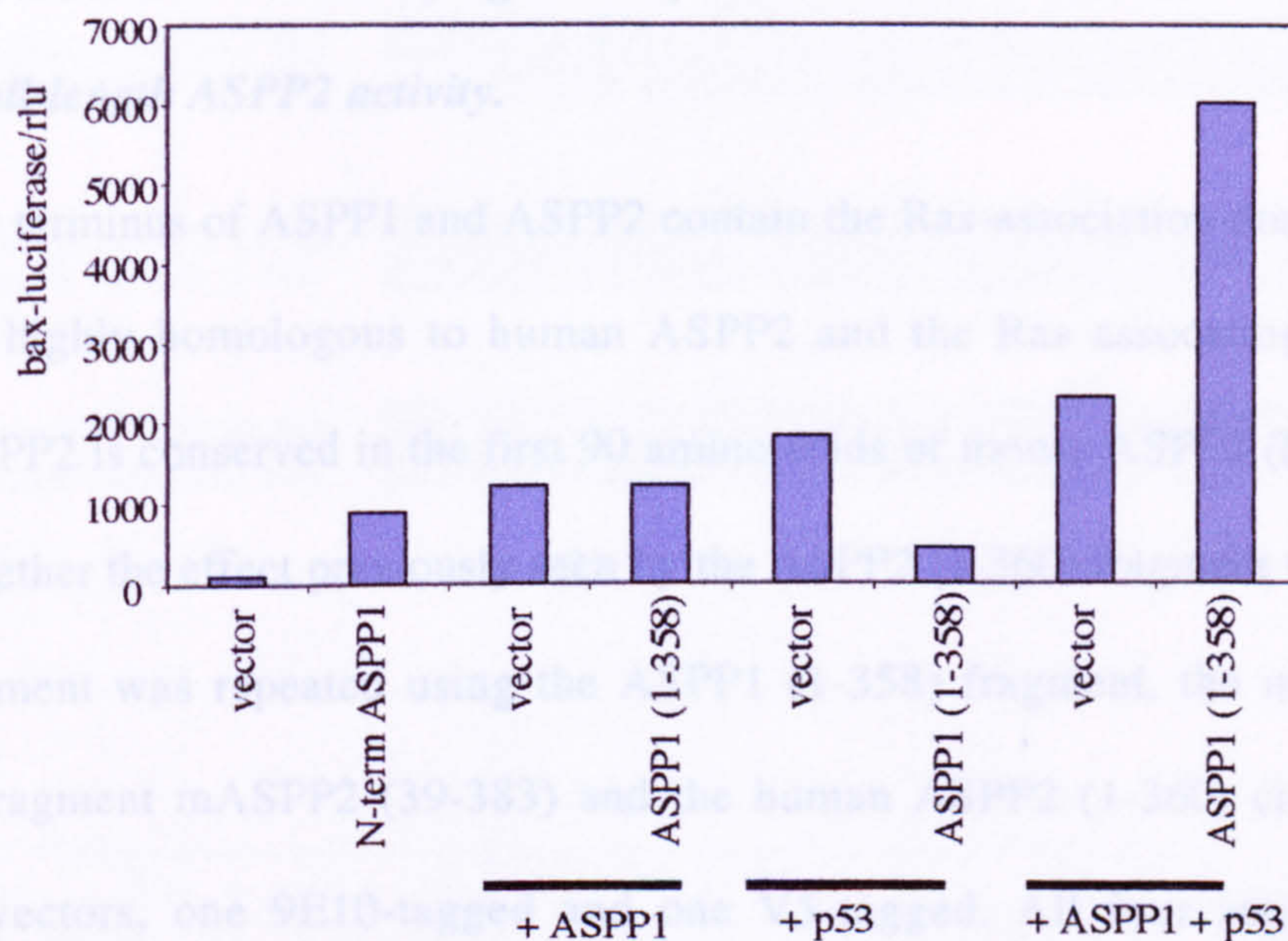
Figure 5.2. *ASPP2 (1-360) increases p53 activity.* 50 ng of pCDNA wild type p53 was transfected into Saos2 cells in the presence or absence of 2 μ g ASPP2 (1-360). 1 μ g Bax-luciferase was transfected per 6 cm dish. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. The mean values were derived from two independent experiments.

5.2.2. The amino terminus of ASPP1 or ASPP2 stimulates p53 activity via full-length ASPP1 and ASPP2

To test whether the effect of the amino terminal fragments of ASPP stimulated p53 directly or via full-length ASPP1 and ASPP2, these fragments were co-transfected full-length ASPP and p53 (figure 5.3). Co-transfection of ASPP1 (1-358) increased p53 and ASPP1 full-length synergy significantly. Similarly, co-transfection of ASPP2 (1-360) increased p53 and ASPP2 synergy.

In this experiment p53 did not synergize greatly with ASPP1 nor ASPP2 when co-transfected in Saos2 cells. However, the presence of both the amino terminus fragment and the full-length ASPP proteins greatly enhanced p53 transactivation activity. The ASPP1 (1-358) and ASPP2 (1-360) stimulation of p53 was dependent on full-length ASPP since the deletion fragments alone did not stimulate p53 to the same extent. ASPP1 (1-358) even inhibited p53 activity in the absence of full-length ASPP1 although this experiment was only performed once and needs to be repeated.

A.



B.

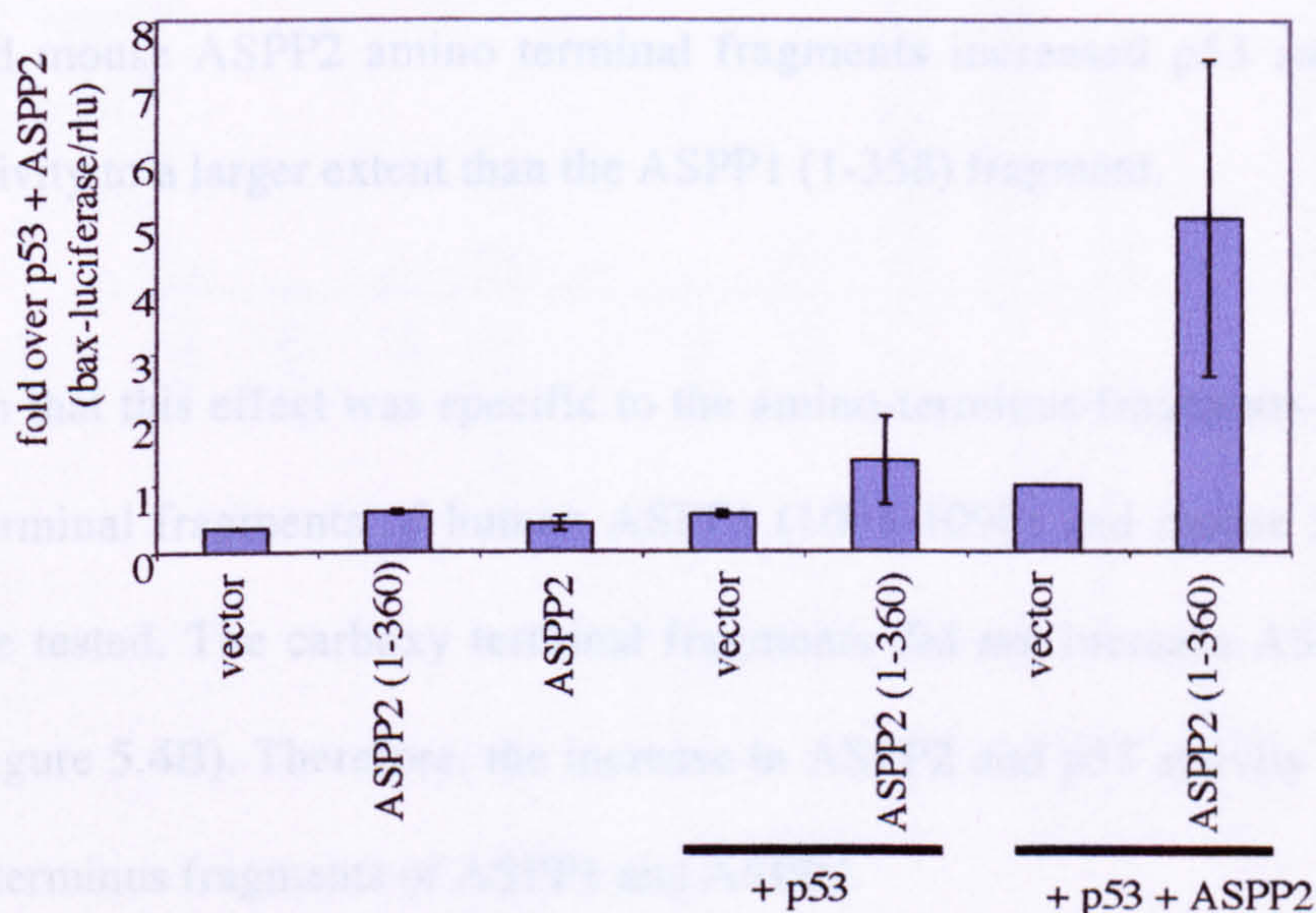


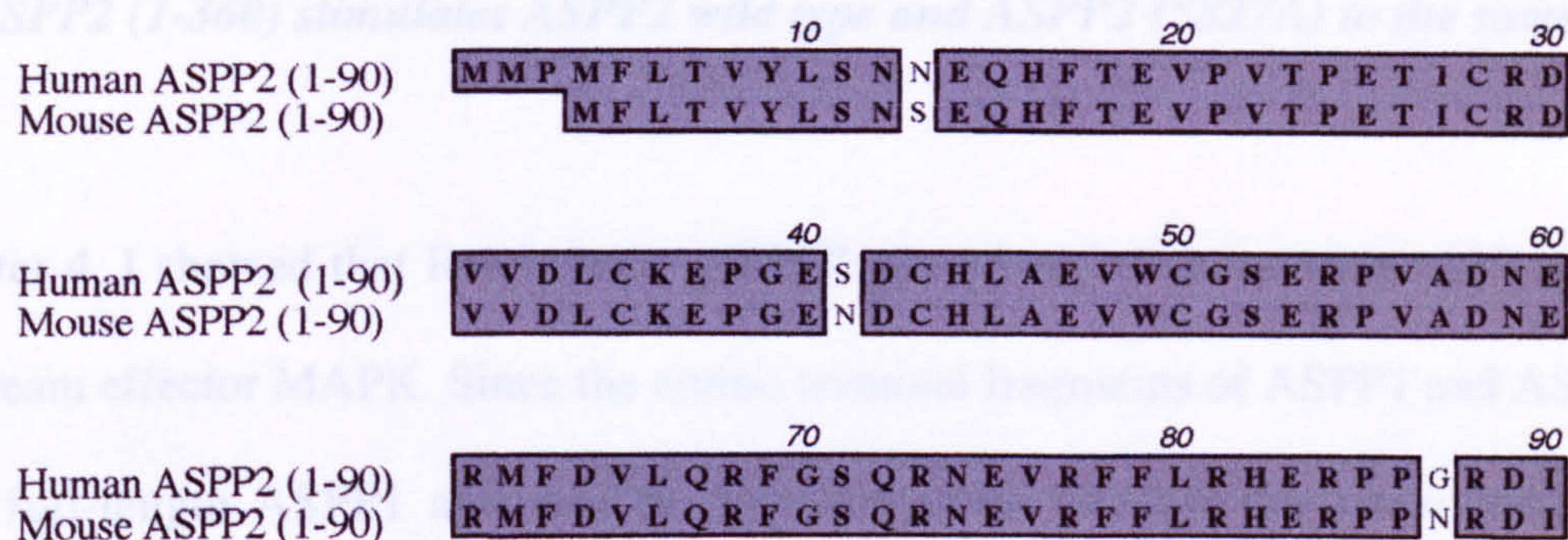
Figure 5.3 The amino-termini of ASPP1 and ASPP2 stimulate p53 and full length ASPP1 and ASPP2 transactivation activity. (A) Saos2 cells were transfected with 4 μ g ASPP1 and 50 ng p53 in the presence or absence of 2 μ g ASPP1 (1-358) in pCDNA expression plasmids. (B) Saos2 cells were transfected with 4 μ g ASPP2, 50 ng p53 and 2 μ g N-terminus ASPP2 as indicated. All samples were co-transfected with bax-luciferase reporter. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. Values shown are fold over p53 + ASPP2. The mean values were derived from two independent experiments.

5.2.3. Only the amino terminus fragments of human and mouse ASPP can stimulate p53 and full-length ASPP2 activity.

The amino terminus of ASPP1 and ASPP2 contain the Ras-association domain. Mouse ASPP2 is highly homologous to human ASPP2 and the Ras associating domain of human ASPP2 is conserved in the first 90 amino acids of mouse ASPP2 (figure 5.4A). To test whether the effect previously seen by the ASPP2 (1-360) fragment was specific, the experiment was repeated using the ASPP1 (1-358) fragment, the mouse amino terminal fragment mASPP2 (39-383) and the human ASPP2 (1-360) cloned in two different vectors, one 9E10-tagged and one V5-tagged. All four amino terminal fragments increased p53 and ASPP2 synergy significantly (figure 5.4B). Both the human and mouse ASPP2 amino terminal fragments increased p53 and full-length ASPP2 activity to a larger extent than the ASPP1 (1-358) fragment.

To confirm that this effect was specific to the amino-terminus fragments of ASPP, the carboxy-terminal fragments of human ASPP1 (1003-1090) and mouse ASPP2 (917-1125) were tested. The carboxy terminal fragments did not increase ASPP2 and p53 synergy (figure 5.4B). Therefore, the increase in ASPP2 and p53 activity is specific to the amino terminus fragments of ASPP1 and ASPP2.

A.



B.

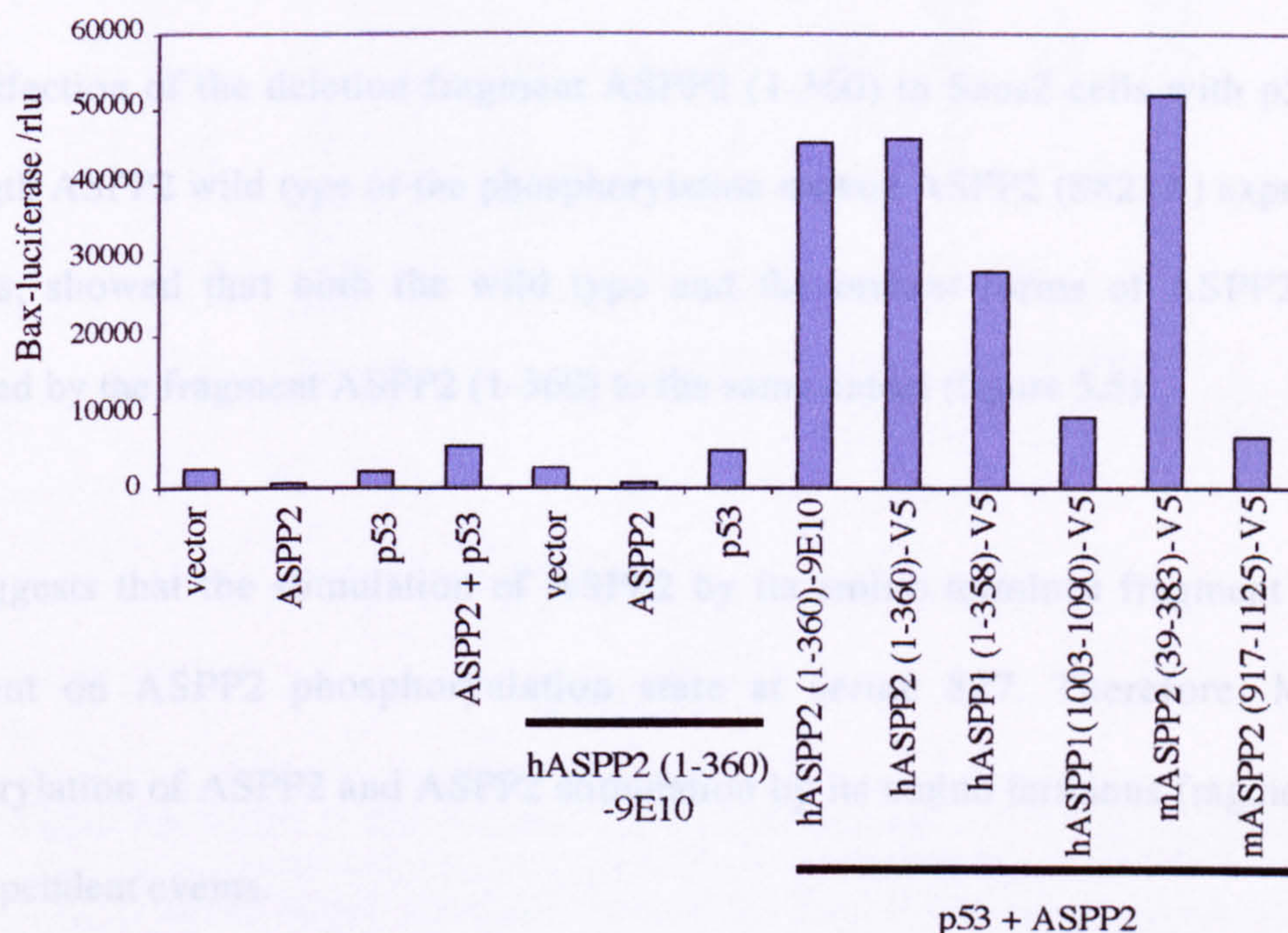


Figure 5.4. Human ASPP1 (1-358), human ASPP2 (1-360) and mouse ASPP2 (59-383) can increase p53 and ASPP2 transactivation activity. (A) Alignment of the ras-association domain of human and mouse ASPP2. (B) Saos2 cells were transfected with 4 μ g of ASPP2 and 50 ng of p53 in the presence of 2 μ g of ASPP1 and ASPP2 deletion fragments. Fragments with the prefix “h” are human and those with the prefix “m” are mouse. All deletions fragments are tagged in their carboxy terminus with either 9E10 or V5 as indicated.

5.2.4. ASPP2 (1-360) stimulates ASPP2 wild type and ASPP2 (S827A) to the same extent.

In chapter 4, I showed that Ras induces ASPP2 phosphorylation on serine 827 via its downstream effector MAPK. Since the amino terminal fragments of ASPP1 and ASPP2 act via full-length ASPP1 and ASPP2, I investigated whether the amino terminal fragment of ASPP2 increases the activity of full-length ASPP2 wild type to the same extent as the phosphorylation mutant ASPP2 (S827A).

Co-transfection of the deletion fragment ASPP2 (1-360) in Saos2 cells with p53 and full-length ASPP2 wild type or the phosphorylation mutant ASPP2 (S827A) expressing plasmids, showed that both the wild type and the mutant forms of ASPP2 were stimulated by the fragment ASPP2 (1-360) to the same extent (figure 5.5).

This suggests that the stimulation of ASPP2 by its amino terminus fragment is not dependent on ASPP2 phosphorylation state at serine 827. Therefore, MAPK phosphorylation of ASPP2 and ASPP2 stimulation by its amino terminus fragment are two independent events.

5.2.5. ASPP2 is the putative substrate of several kinases

Previous work in this thesis has shown that ASPP2 is a substrate for MAPK phosphorylation. Phosphorylation is a post-translational modification that allows a rapid

induction of a protein's activity and allows a signal to be amplified as it passes through a signalling cascade. Phosphorylation is therefore a common form of regulation, particularly frequent for proteins involved in cell cycle control.

It was of interest to determine whether ASPP2 could be phosphorylated by kinases other than MAPK. Figure 5.5 shows the results of a luciferase assay using the

web-site <http://caslab.cba.hawaii.edu/kinase/> to predict the phosphorylation of ASPP2. ASPP2 is a putative substrate for a number of different kinases. Figure 5.6 shows the results of a luciferase

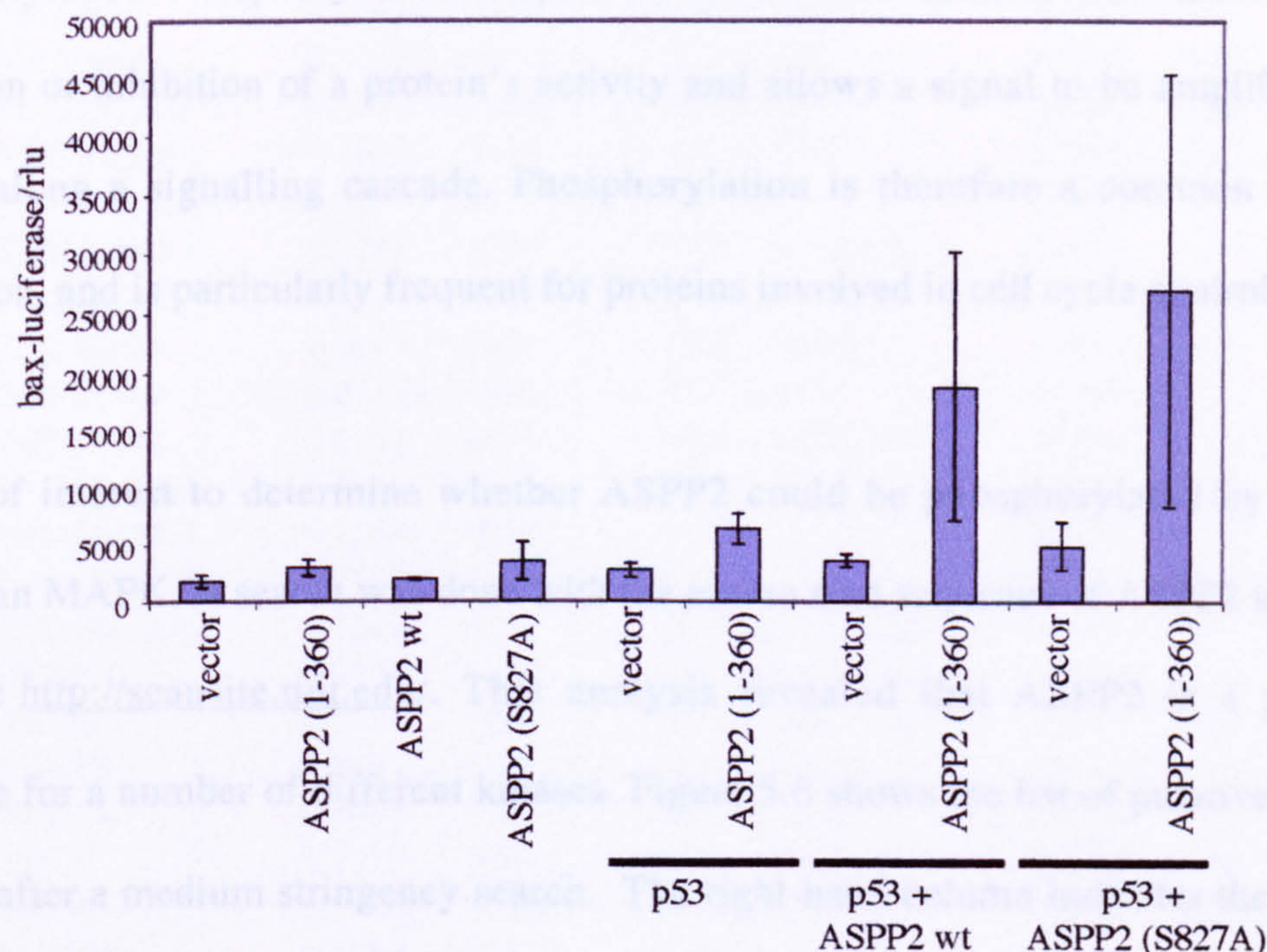
assay after a medium stringency search. The results show that ASPP2 is a putative substrate for a number of different kinases. Figure 5.6 shows the results of a luciferase

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Figure 5.5. *ASPP2 (1-360) stimulates ASPP2 wild type and ASPP2 (S827A).* Saos2 cells were transfected with 4 μ g ASPP2 wild type or ASPP2 (S827A), 50 ng of p53 and 2 μ g of ASPP2 (1-360) expression plasmids. All samples were co-transfected with bax-luciferase. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. The mean values were derived from two independent experiments.



5.2.5. ASPP2 is the putative substrate of several kinases

Previous work in this thesis has shown that ASPP2 is a substrate for MAPK phosphorylation. Phosphorylation is a post-translational modification that allows a rapid induction or inhibition of a protein's activity and allows a signal to be amplified as it passes along a signalling cascade. Phosphorylation is therefore a common form of regulation, and is particularly frequent for proteins involved in cell cycle control.

It was of interest to determine whether ASPP2 could be phosphorylated by kinases other than MAPK. A search was done with the amino acid sequence of ASPP2 using the web-site <http://scansite.mit.edu/>. This analysis revealed that ASPP2 is a putative substrate for a number of different kinases. Figure 5.6 shows the list of putative ASPP2 kinases after a medium stringency search. The right-hand column indicates the residue within the consensus sequence that is the putative phosphorylation site for a particular kinase. In some cases, a particular kinase has several potential phosphorylation sites in ASPP2. The middle column shows the probability of the highlighted residue being phosphorylated: the lower the percentile, the higher the probability that ASPP2 might be phosphorylated by that particular kinase at that particular site. It can be seen that kinases Abl, MAPK1, PDK1, PKA and PKC ϵ have a high probability of phosphorylating ASPP2.

kinase	percentile	position	kinase	percentile	position
Abl kinase	0.593%	Y645	Nck SH2	0.892%	Y869
Abl SH3	0.095%	P872	Nck 2nd SH3	0.416%	P868
Abl SH4	0.414%	P500	p38 MAPK	0.220%	S673
Akt kinase	0.824%	S1126	p38 MAPK	0.668%	S698
Amphiphysin SH3	0.680%	P360	p38 MAPK	0.668%	T586
ATM kinase	0.466%	T614	p38 MAPK	0.994%	S556
ATM kinase	0.588%	S423	p85 SH3 mode2	0.940%	P360
ATM kinase	0.779%	S96	PDK1 Binding A	0.062%	D1067
Cdc2 Kinase	0.324%	S673	PDK1 Binding A	0.629%	D461
Cdc2 Kinase	0.754%	S698	PDK1 Binding B	0.984%	T614
Cdk5 kinase	0.875%	S698	PDZ class 1	0.146%	S1126
Cdk5 kinase	0.875%	S673	PDZ (nNOS) class 1	0.084%	S1126
Cortactin SH3	0.036%	P503	PKA	0.111%	S737
Cortactin SH3	0.156%	P870	PKA	0.890%	S736
Cortactin SH3	0.278%	P696	PKA	0.890%	S296
Cortactin SH3	0.324%	P360	PKCalpha/beta/gamma	0.631%	T474
Crk SH2	0.957%	Y869	PKCalpha/beta/gamma	0.863%	S100
Crk SH3	0.957%	Y869	PKC epsilon	0.031%	S737
DNA PK	0.029%	P500	PKC epsilon	0.034%	T758
DNA PK	0.595%	S96	PKC epsilon	0.421%	T603
Erk1	0.111%	S698	PKC epsilon	0.558%	S458
Grb2 SH2	0.368%	Y710	PKC mu	0.432%	S726
Grb2 SH3	0.342%	P872	PKC mu	0.535%	T757
Grb2 SH3	0.922%	P870	PKC mu	0.615%	S934
Intersectin SH3A	0.145%	P503	PKC zeta	0.348%	S1126
Itk SH3	0.263%	P500	PLCg N-terminal SH2	0.438%	Y791
Itk SH3	0.565%	P357	PLCg SH3	0.967%	P871
Itk SH3	0.845%	P578	Shc PTB	0.596%	Y869
Itk SH3	0.933%	P871	Src SH3	0.551%	P500

Figure 5.6. Putative ASPP2 kinases. The amino acid sequence of ASPP2 was used to search for putative phosphorylation sites of kinases with the scansite search engine. The search was done at medium stringency. The percentile shows the probability of the kinase phosphorylating ASPP2 at that site; the higher the percentage, the lower the probability. The columns on the right of the tables show the position and the residue of ASPP2 that could phosphorylated by the given kinase.

5.2.6. ASPP2 is phosphorylated in vitro by p90rsk, PKA and p38 SAPK

A selection of kinases identified by the search (PKA, PKB/Akt, p38SAPK/MAPK, and MAPK1) were examined for their potential to phosphorylate ASPP2 *in vitro*. In addition, two other kinases, p70 S6 kinase and p90 rsk, that were not identified by the search were also analyzed for their potential to act as ASPP2 kinases. These kinases were chosen for the *in vitro* phosphorylation assay due to the fact that they were readily available in the laboratory of Dario Alessi (University of Dundee).

The recombinant GST-ASPP2 (693-1128) fragment was used as a substrate. This contains the majority of the putative phosphorylation sites. Histone 2B was used as a positive control as it is a substrate for a wide range of kinases *in vitro*, and MAPK1 was also used as a positive control for ASPP2 phosphorylation. Figure 5.7 shows that ASPP2 (693-1128) was phosphorylated by MAPK1 as expected, as well as p90, PKA and p38SAPK. PKB was unable to phosphorylate the ASPP2 fragment although it was still active, as seen by the histone 2B phosphorylation. It remained inconclusive whether p70 S6K could phosphorylate ASPP2 *in vitro* as it did not phosphorylate its positive control histone 2B.

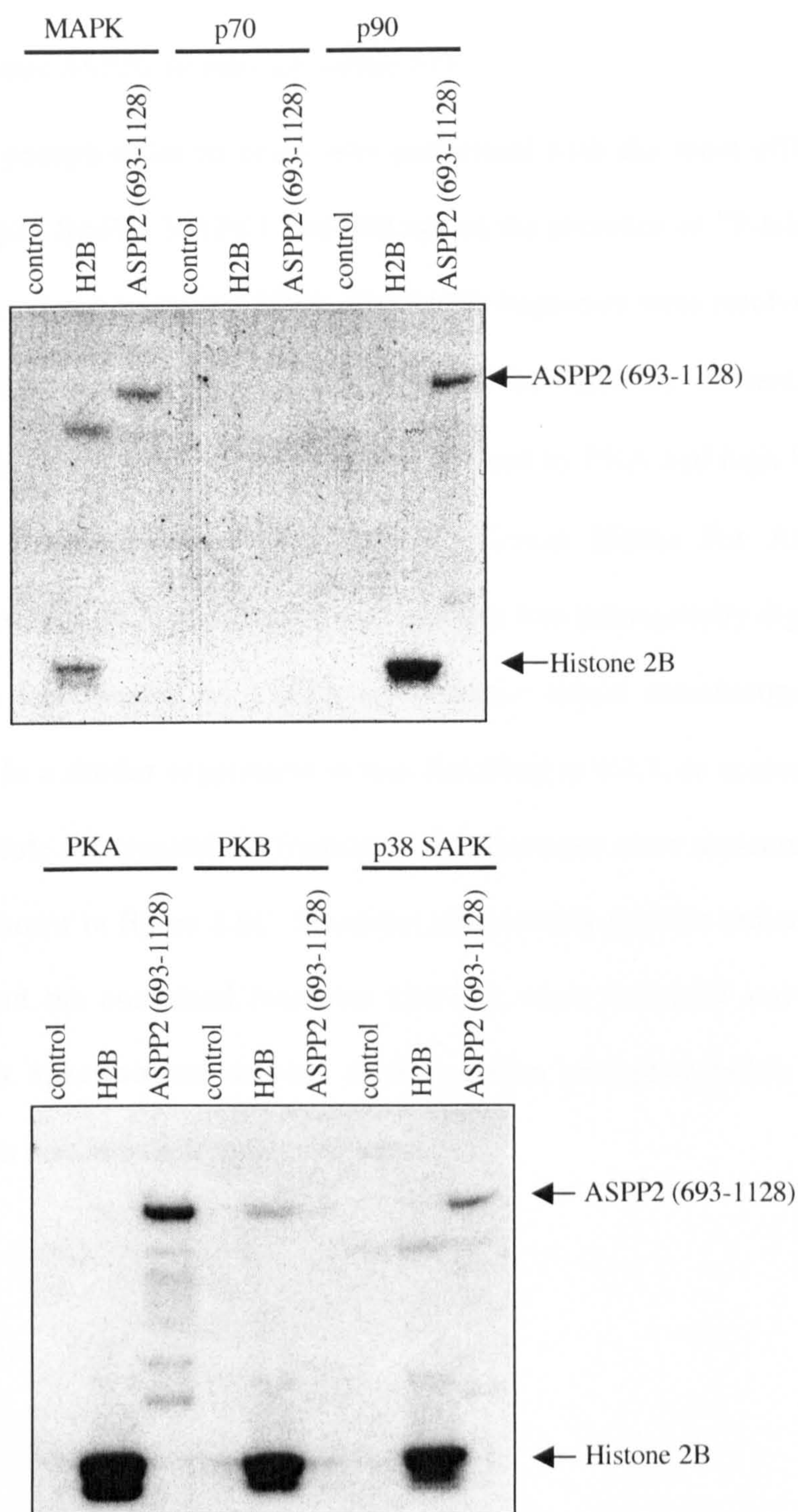


Figure 5.7 *ASPP2 (693-1128) is phosphorylated in vitro by a variety of kinases.* A GST-ASPP2 (693-1128) fragment was expressed in BL21 cells and purified as described in Materials & Methods. ASPP2 (693-1128) was used as a substrate for an *in vitro* phosphorylation assay by the kinases MAPK, p70, p90, PKA, PKB and p38SAPK. As a negative control no substrate was used and Histone 2B (H2B) was the substrate for the positive control. ^{32}P -labelled ATP was added to the kinase assay and the labelled proteins were resolved on SDS-PAGE gels and visualized by autoradiograph.

5.2.7. PKA phosphorylates ASPP2 in vitro on serine 737

A large-scale *in vitro* phosphorylation assay was performed with the most efficient ASPP2 kinases: PKA, p38 SAPK, MAPK1 and p90 rsk, in the presence of ^{32}P -labelled ATP (figure 5.8A). The phosphorylated ASPP2 (693-1128) fragments were resolved on an SDS-PAGE gel and excised following which their radioactivity content was measured (figure 5.8B). The ASPP2 fragment phosphorylated by PKA had high levels of incorporated ^{32}P , suggesting that PKA is an efficient kinase for ASPP2 phosphorylation. The excised PKA-phosphorylated fragment was subsequently digested with trypsin and then fractionated on a high performance liquid chromatography (HPLC) C-18 column. In a similar experiment to that described in 4.2.3, an acetonitrile gradient was used to elute the trypsinized fragments. All fractions were measured for their radioactivity as shown in figure 5.8C. Fractions representing the two radioactive peaks, fraction 103 and the combined fractions 124/125, were collected and were analyzed by the mass spectrometer service at the protein phosphorylation unit, university of Dundee, to measure their molecular mass.

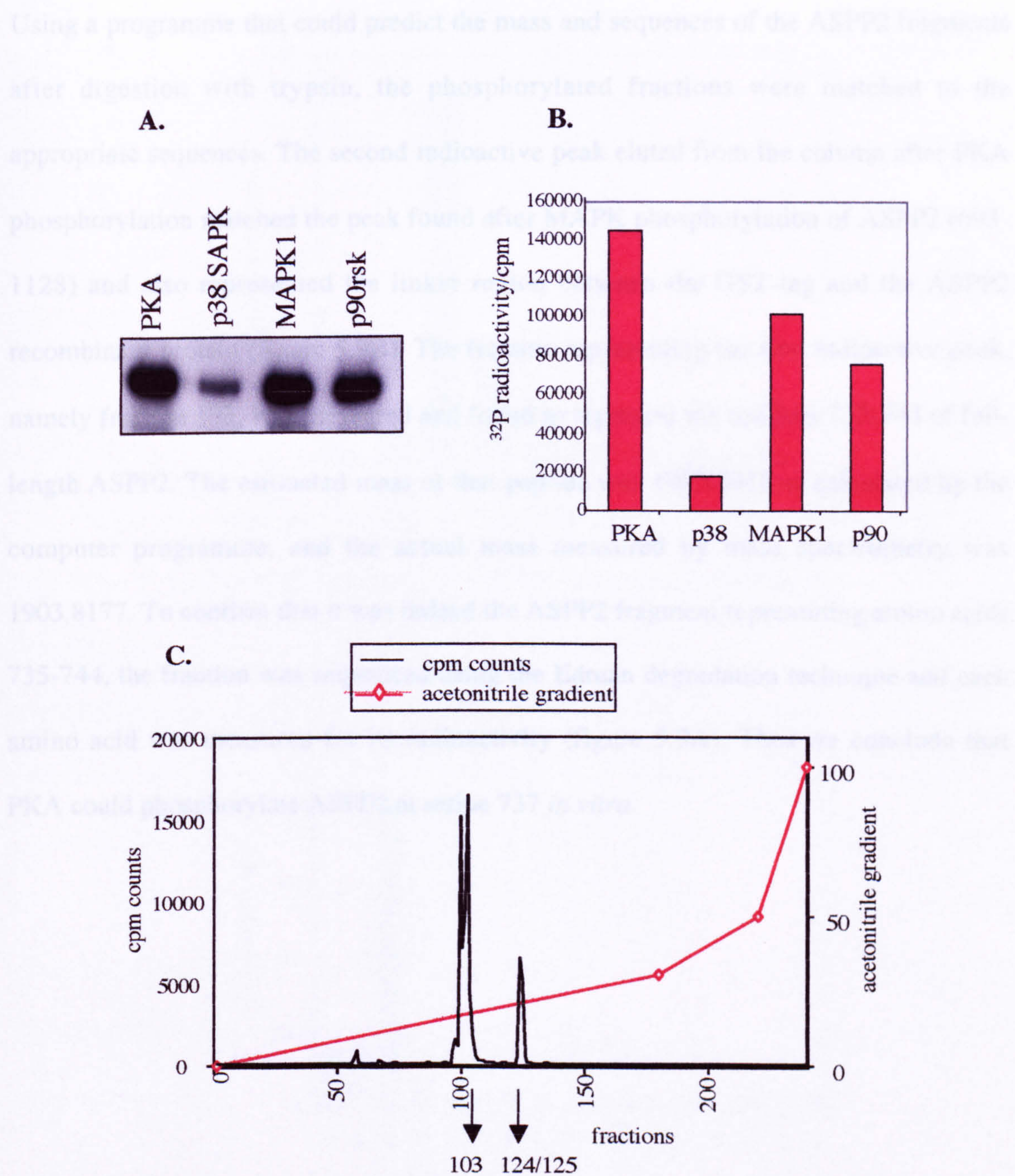


Figure 5.8. PKA phosphorylates ASPP2 *in vitro* with high activity. (A) large-scale *in vitro* phosphorylation assay of ASP2 (693-1128). The intensity of the bands was quantified and is shown in (B). The PKA-phosphorylated ASPP2 (693-1128) fragment was trypsinized and the peptides separated with a high performance liquid chromatography (HPLC) using increasing amounts of acetonitrile. The fractions were collected and their radioactivity measured, as shown by the black peaks. The radioactive peaks represented fractions 103 and 124/125.

Using a programme that could predict the mass and sequences of the ASPP2 fragments after digestion with trypsin, the phosphorylated fractions were matched to the appropriate sequences. The second radioactive peak eluted from the column after PKA phosphorylation matched the peak found after MAPK phosphorylation of ASPP2 (693-1128) and also represented the linker region between the GST-tag and the ASPP2 recombinant protein (figure 5.9B). The fraction representing the first radioactive peak, namely fraction 103, was measured and found to represent the residues 735-744 of full-length ASPP2. The estimated mass of that peptide was 1903.8915 as calculated by the computer programme, and the actual mass measured by mass spectrometry was 1903.8177. To confirm that it was indeed the ASPP2 fragment representing amino acids 735-744, the fraction was sequenced using the Edman degradation technique and each amino acid was measured for its radioactivity (figure 5.9A). Thus we conclude that PKA could phosphorylate ASPP2 at serine 737 *in vitro*.

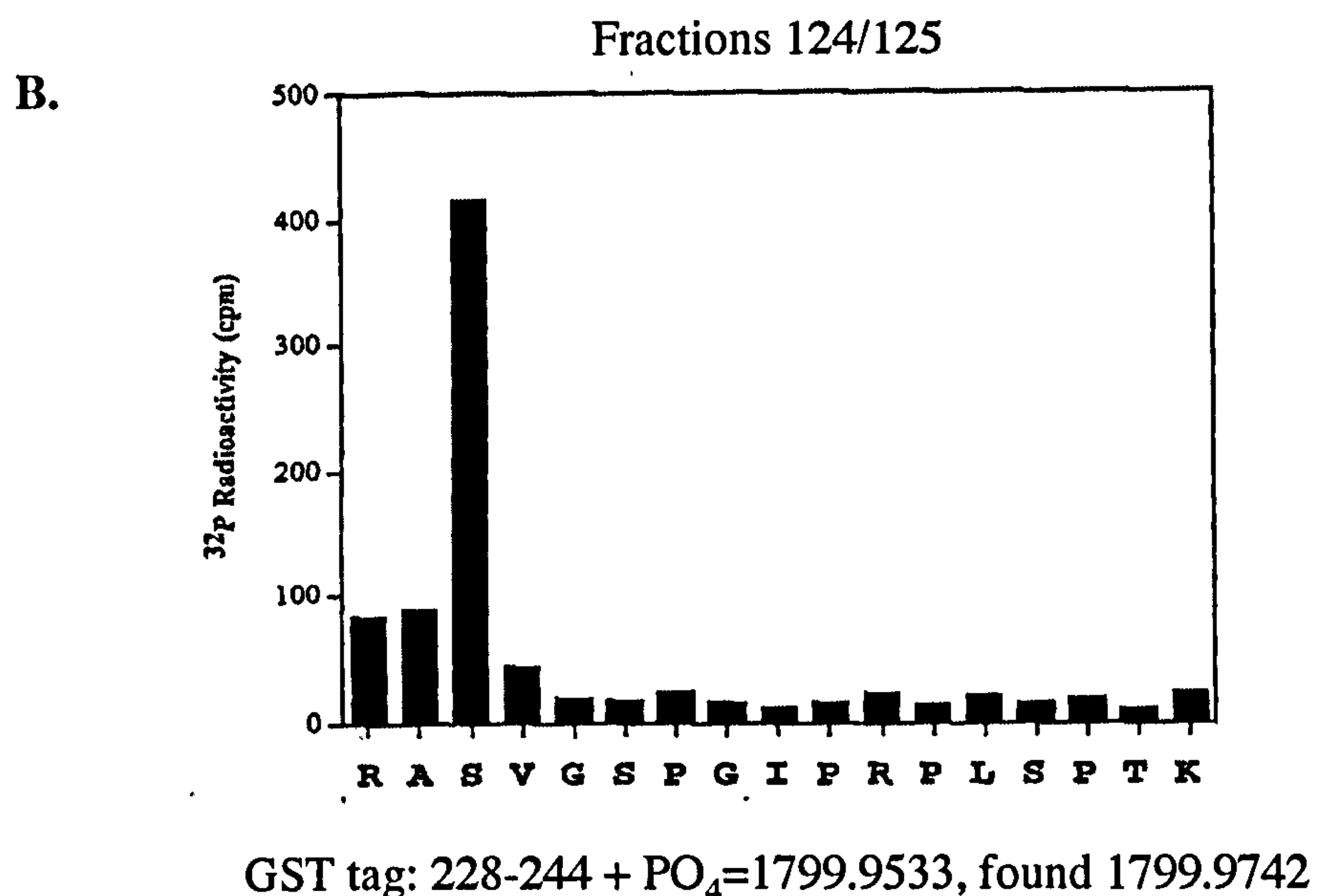
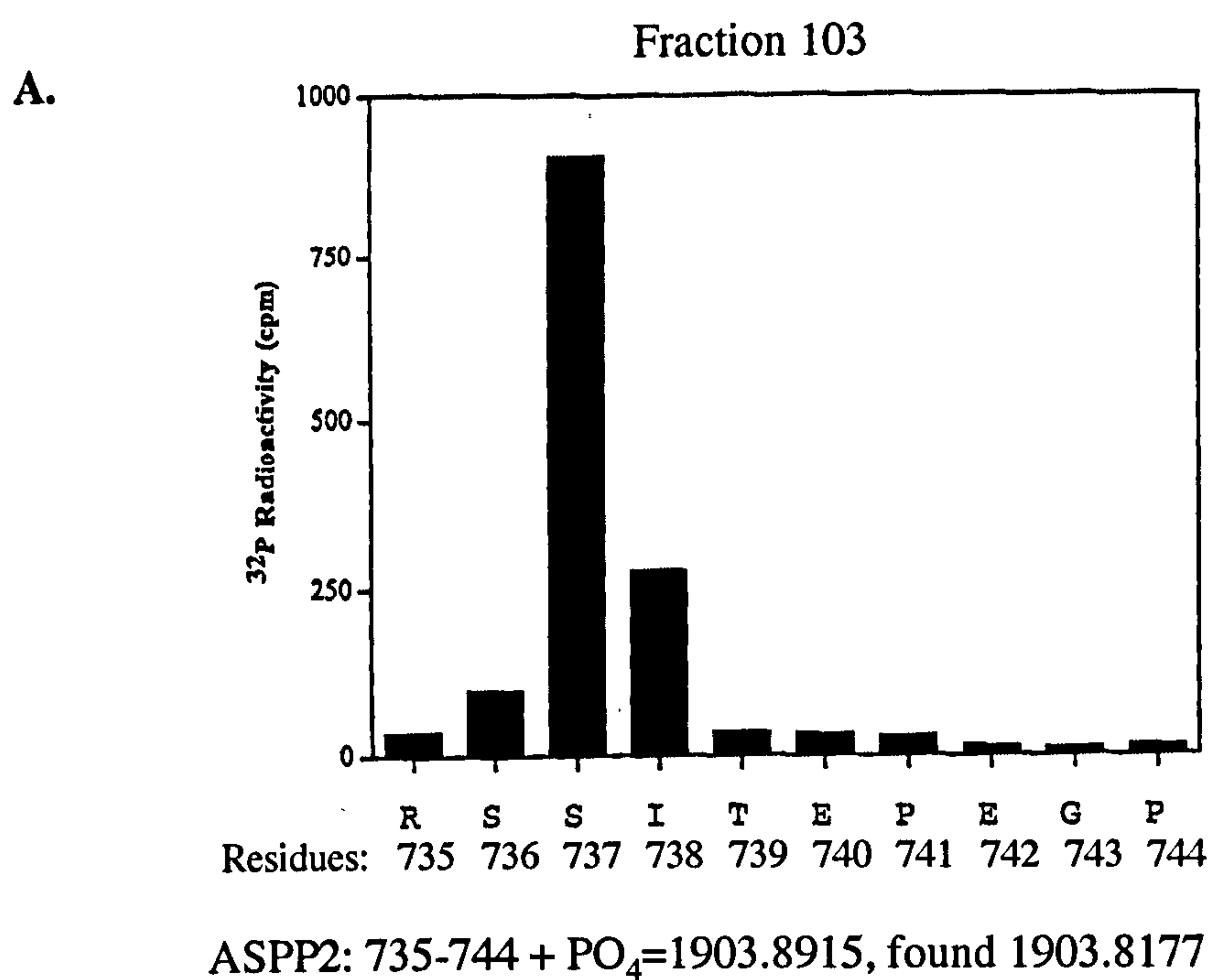


Figure 5.9. PKA phosphorylates ASPP2 (693-1128) *in vitro* on residue 737. The radioactive fractions were collected and measured by mass spectrometry. (A) Mass spectrometry analysis and Edman degradation showed that fraction 103 contained the serine 737 phosphorylated by PKA. The graph shows each residue from the fraction labelled with its amino acid composition and its sequence number. The values shown under the graph represents the expected mass of the fraction after phosphorylation (PO₄) and the actual ("found") mass. (B) Fractions 124/125 represented the linker region between the GST-tag and the ASPP2 fragment, and was phosphorylated on residue 230. As with A, the values shown under the graph represents the the expected mass of the fraction after phosphorylation (PO₄) and the actual ("found") mass.

5.2.8. ASPP2 (S736,737A) phosphorylation mutant is as active as ASPP2 wild type

To test the effect of PKA on ASPP2 *in vivo*, an ASPP2 phosphorylation mutant was constructed with the two serines at positions 736 and 737 mutated to alanine (figure 5.10A). Since PKA was shown to phosphorylate serine 737 *in vitro*, this was thought to inhibit any effect PKA might have on ASPP2 activity.

5.2.8.1. Construction of ASPP2 (S736,737A) phosphorylation mutant

The mutant was constructed by site directed mutagenesis using the carboxy-terminal fragment of ASPP2 (693-1128) in a pCDNA3 vector as a template (figure 5.10B). A titration of the template was used for PCR and the mutated carboxy terminal fragment of ASPP2 was purified (figure 5.10D). The amino terminus fragment of ASPP2 (1-692) was isolated from a full-length ASPP2 construct by EcoRI digestion. It was then inserted into the EcoRI-digested mutant construct, 5' of the carboxy terminal fragment of ASPP2 (figure 5.10E). This resulted in a full-length mutant ASPP2, as shown schematically in figure 5.10C. The full-length APP2 (S736,737A) mutant was sequenced and shown to contain the wanted mutation but no other change in sequence (data not shown).

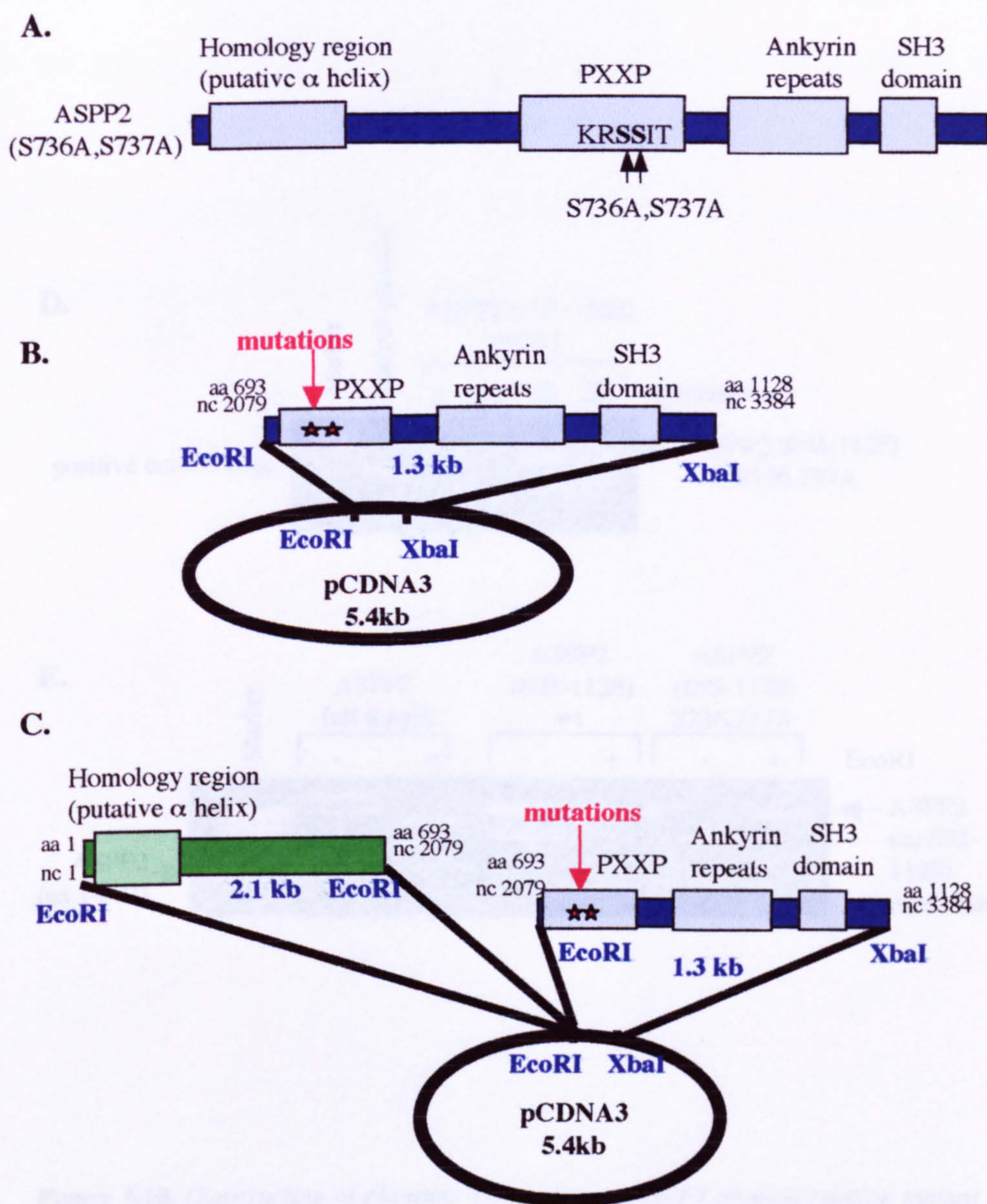


Figure 5.10. Construction of plasmids expressing an ASPP2 phosphorylation mutant at the PKA site. (A) Schematic representation of plasmids encoding mutant ASPP2. The homologous region in the amino terminus is shown, as is the proline rich region (PXXP), the ankyrin repeats and the SH3 domain. The putative PKA phosphorylation site that was mutated to alanine (serines 736 and 737) is shown. The mutations were introduced by site-directed mutagenesis in the ASPP2 fragment in a pCDNA vector (described in Materials & Methods) representing amino acids 693-1128 and nucleotides 2079-3384, as shown schematically in figure (B). (C) Schematic representation of the amino-terminus fragment (amino acids 1-693, representing nucleotides 1-2079) subsequently added to the mutated carboxy-terminus fragment.

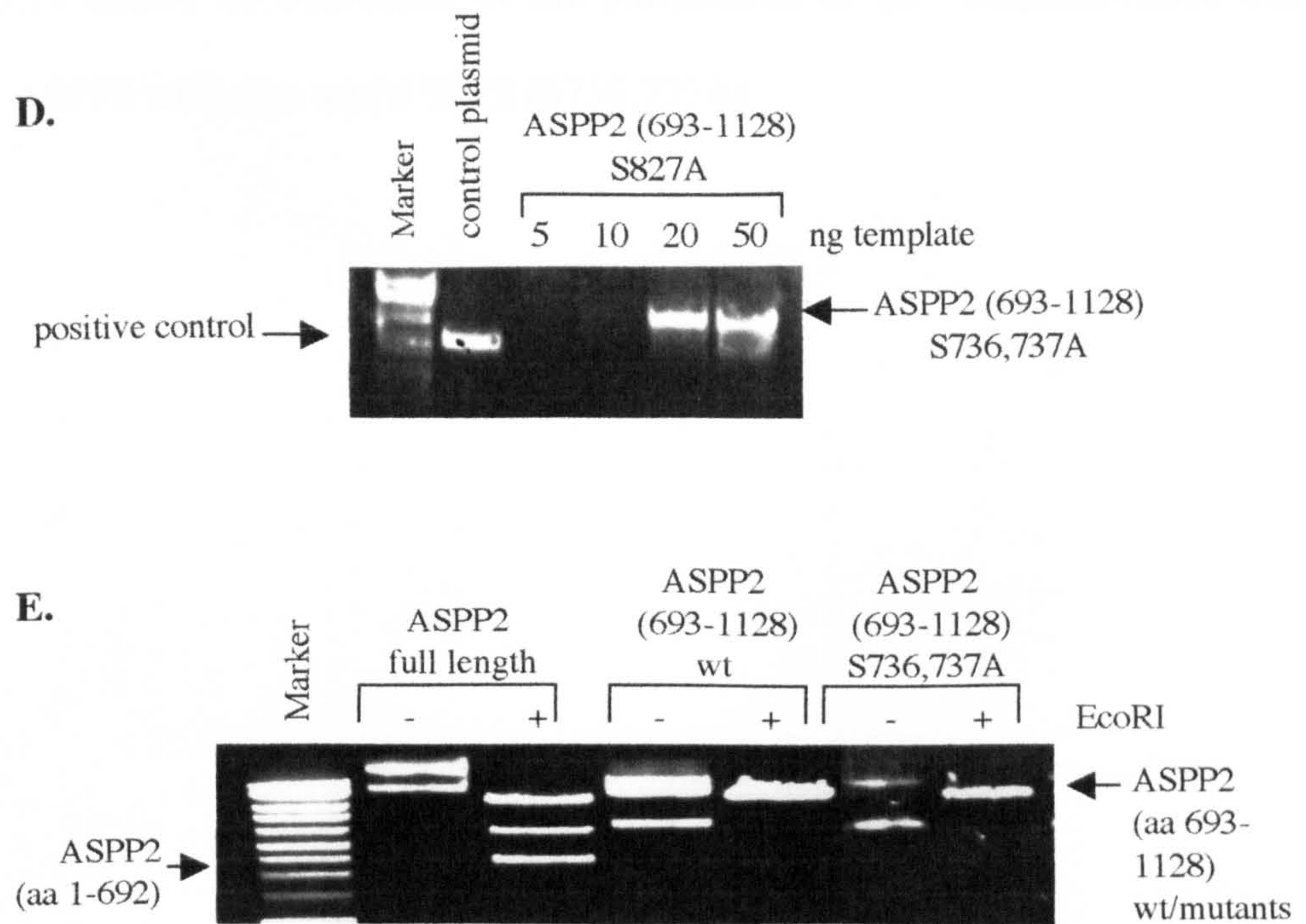


Figure 5.10. *Constructing of plasmids expressing an ASPP2 phosphorylation mutant at the PKA site, continued.* (D) Site-directed mutagenesis was performed by PCR on ASPP2 (693-1128) in a pCDNA template to produce the S736,737A mutant. The first lane shows the 1 kb DNA marker and the second lane the control pBluescript template. The last four lanes show mutagenesis PCR products performed with a titration of template, as indicated (E) Full length ASPP2 was digested with EcoRI as were the mutant and wild type ASPP2 fragments (nc 2079-3384, representing amino acids 693-1128). All digests were resolved on a 1% agarose gel. The digested fragment of ASPP2 representing the nucleotides 1-2079 (amino acids 1-693) was purified from the gel. The digested bands representing the wild-type and mutant fragments (nucleotides 2079-3384 representing amino acids 693-1128) were also purified.

5.2.8.2. ASPP2 (S736,737A) is as active as ASPP2 wild type in inducing p53

To test the activity of ASPP2 (S736,737A), the phosphorylation mutant was co-transfected with p53 and compared to ASPP2 wild type co-transfection with p53. Figure 5.11 shows no difference in the stimulation of p53 transactivation activity between ASPP2 wild type and ASPP2 (S736,737A).

5.2.9. Forskolin stimulates both ASPP2 wild type and ASPP2 (S736,737A) activity

As seen with the ASPP2(S736,737A) mutant defective in the MAPK phosphorylation site, basal activity could be similar to that of wild type ASPP2 and only under certain conditions was a difference perceptible in the mutant and wild type activity. If PKA phosphorylates ASPP2 *in vivo*, the situation where the difference between ASPP2 wild type and ASPP2 (S736,737A) would be most apparent would be those in which

endogenous PKA is activated.

Forskolin, a well-known activator of PKA, is also an adenylyl cyclase to release increasing amounts of cAMP resulting in PKA activation (Strydom and Lindley, 1981; Segall and Strydom, 1991).

Saos2 cells were transfected with p53 and ASPP2 wild type or ASPP2 (S736,737A) in

the presence or absence of forskolin. Forskolin increased p53 transactivation activity on

the bax-luciferase reporter markedly. Stimulation by forskolin was via ASPP2 as it only

had a small effect on p53 activity alone.

Figure 5.11. ASPP2 wild type and ASPP2 (S736A,S737A) have similar activity. Saos2 cells were transfected with 50 ng of p53 and 4 μ g of ASPP2 wild type or ASPP2 (S736,737A). Bax-luciferase was co-transfected in all samples. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. The luciferase counts are shown. The p53 values were arbitrarily taken as 1.0 to see the effect of ASPP2 on its activity.

able to extensively stimulate p53 and ASPP2 (S736,737A) phosphorylation.

Although it seemed from the luciferase counts that ASPP2 (S736,737A) was not

stimulated by forskolin to the same extent as ASPP2 wild type was, this was due to the

fact that in this particular experiment the mutant ASPP2 did not synergize with p53 as

effectively as did wild type ASPP2. To determine whether there was any difference in

forskolin stimulation of ASPP2 wild type and ASPP2 mutant, the values of p53 and

ASPP2 were arbitrarily put as 1.0 (figure 5.12B). This allowed analysis of effect of

5.2.9. Forskolin stimulates both ASPP2 wild type and ASPP2 (S736,737A) activity

As seen with the ASPP2 (S827A) mutant defective in the MAPK phosphorylation site, basal activity could be similar to that of wild type ASPP2 and only under certain conditions can a difference be perceived in the mutant and wild type activity. If PKA phosphorylates ASPP2 *in vivo*, the situation where the difference between ASPP2 wild type and ASPP2 (S736,737A) would be most apparent would be those in which endogenous PKA is activated.

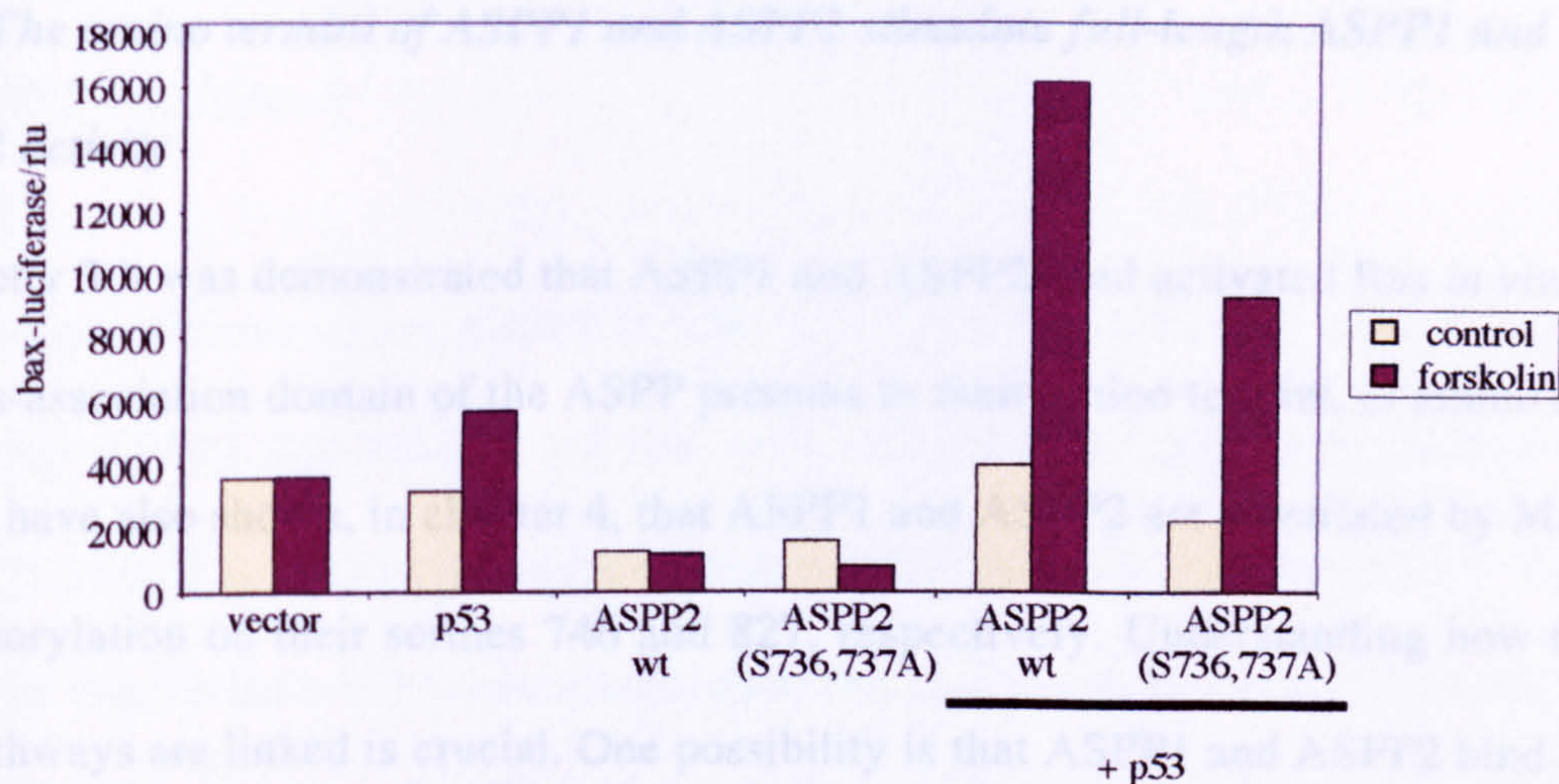
Forskolin is a well-known activator of PKA. It stimulates adenylate cyclase to release increasing amounts of cAMP resulting in PKA activation (Metzger and Lindner, 1981; Seamon and Daly, 1981). Forskolin was therefore used to stimulate endogenous PKA. Saos2 cells were transfected with p53 and ASPP2 wild type or ASPP2 (S736,737A) in the presence or absence of forskolin. Forskolin increased p53 transactivation activity on the bax-luciferase reporter markedly. Stimulation by forskolin was via ASPP2 as it only had a slight stimulatory activity on p53 alone but a considerable stimulatory effect on p53 and ASPP2 (figure 5.12A). This suggested that PKA is an upstream, positive regulator of ASPP2. As well as stimulating p53 and ASPP2 wild type, forskolin was able to extensively stimulate p53 and ASPP2 (S736,737A) phosphorylation mutant.

Although it seemed from the luciferase counts that ASPP2 (S736,737A) was not stimulated by forskolin to the same extent as ASPP2 wild type was, this was due to the fact that in this particular experiment the mutant ASPP2 did not synergize with p53 as effectively as did wild type ASPP2. To determine whether there was any difference in forskolin stimulation of ASPP2 wild type and ASPP2 mutant, the value of p53 and ASPP2 were arbitrarily put as 1.0 (figure 5.12B). This allowed analysis of effect of

forskolin on p53 and ASPP2 activity. Indeed, forskolin stimulated ASPP2 wild type and ASPP2 (S736A, S737A) equally well and showed no preference for ASPP2 wild type. Thus, although PKA can stimulate ASPP2, it does so via a mechanism independent of phosphorylation on residues 736 and 737.

5.3. Discussion

A.



B.

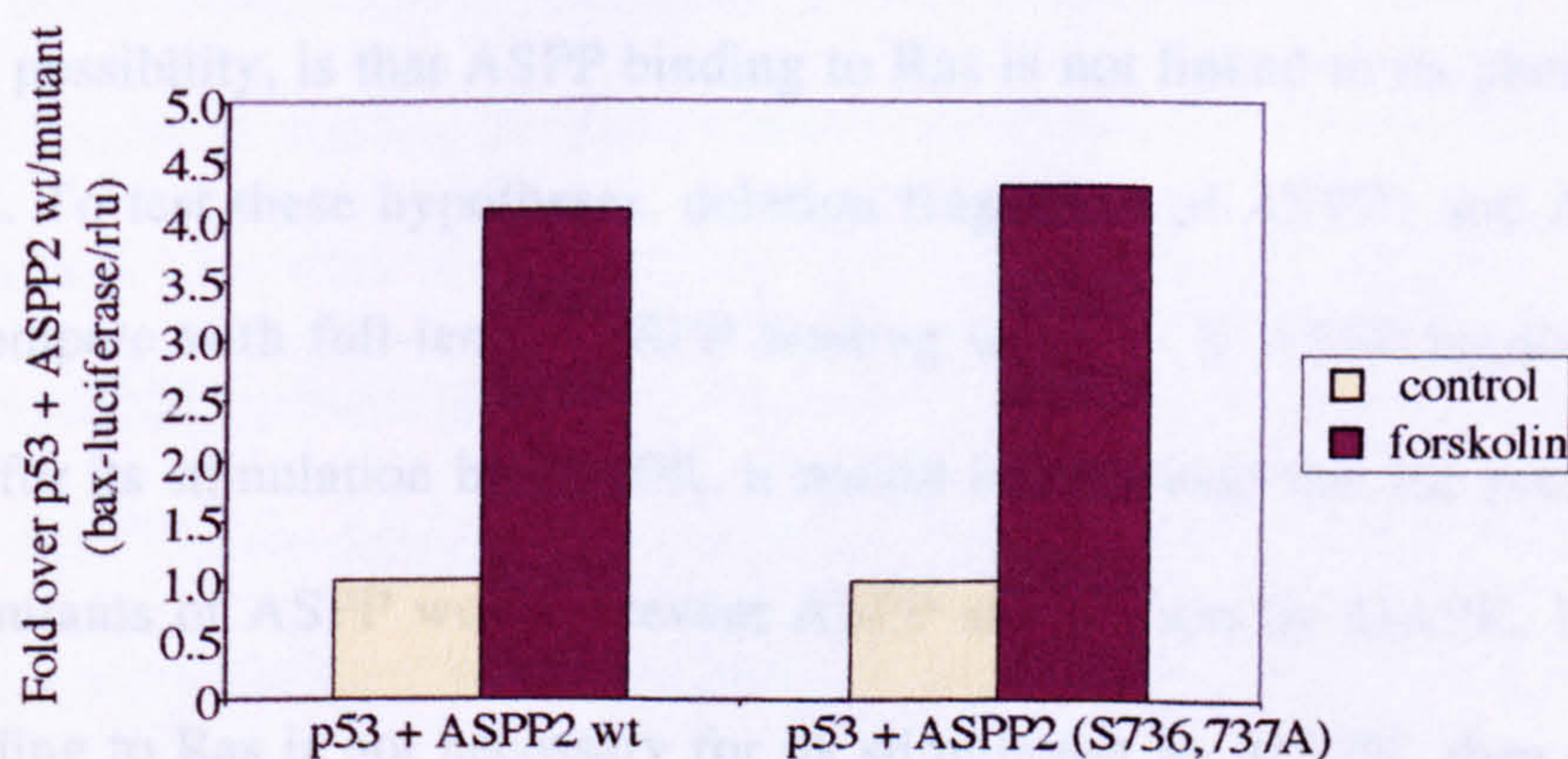


Figure 5.12. The PKA activator forskolin stimulates ASPP2 wild type and ASPP2 (S736,737A). (A) Saos2 cells were transfected with 50 ng of p53 and 4 μ g of ASPP2 wild type or ASPP2 (S736,737A). The cells were treated with 20 μ M forskolin or DMSO as a control. Bax-luciferase reporter was co-transfected in all samples. The cells were harvested and lysed with the luciferase lysis buffer (Promega) and a luciferase assay carried out. The luciferase counts are shown. (B) The value of p53 and ASPP2 was arbitrarily taken as 1.0 to see the effect of forskolin on its activity.

5.3. Discussion

5.3.1. The amino termini of ASPP1 and ASPP2 stimulate full-length ASPP1 and ASPP2 activity

In chapter 3 it was demonstrated that ASPP1 and ASPP2 bind activated Ras *in vivo* via the Ras-association domain of the ASPP proteins in their amino termini, at amino acids 1-90. I have also shown, in chapter 4, that ASPP1 and ASPP2 are stimulated by MAPK phosphorylation on their serines 746 and 827, respectively. Understanding how these two pathways are linked is crucial. One possibility is that ASPP1 and ASPP2 bind Ras-GTP, following which the Raf-MAPK pathway is activated and ASPP1 and ASPP2 are phosphorylated resulting in increased activity. Alternatively, ASPP1 and ASPP2 may be phosphorylated by MAPK, following which they bind activated Ras. Yet a third, albeit less likely possibility, is that ASPP binding to Ras is not linked to its phosphorylation by MAPK. To test these hypotheses, deletion fragments of ASPP1 and ASPP2 were used to compete with full-length ASPP binding to Ras. If ASPP binding to Ras is necessary for its stimulation by MAPK, it would be expected that the presence of the deletion mutants of ASPP would prevent ASPP stimulation by MAPK. If, however, ASPP binding to Ras is not necessary for its stimulation by MAPK, then the deletion fragments would have no effect on ASPP stimulation by MAPK.

Unexpectedly, ASPP2 (1-360) fragment increased p53 activity at least two fold. This stimulation of p53 was probably via endogenous ASPP since the ASPP2 (1-360) stimulation of p53 was significantly enhanced in the presence of exogenous ASPP2: whereas ASPP2 (1-360) stimulated p53 alone two-fold, it stimulated p53 and ASPP2 together more than five-fold. Both ASPP1 (1-358) and ASPP2 (1-360) could stimulate exogenous ASPP1 and ASPP2, respectively. The amino terminus fragment of ASPP2

(1-360) could stimulate full-length ASPP2 independently of MAPK phosphorylation since no difference was observed between the ASPP2 (S827A) phosphorylation mutant and ASPP2 wild type.

In all the experiments where ASPP1 (1-358) and ASPP2 (1-360) were able to increase full-length ASPP1 and ASPP2 activity, p53 did not synergize significantly with ASPP1/2. This lack of synergy might be linked to the fact that a significant amount of DNA was transfected – it has often been observed that the more DNA transfected, the lower the synergy. Although ASPP1/2 were not as active on their own, they were still responsive to the amino terminal fragments.

The effect of the amino terminal fragment of ASPP1 and ASPP2 is not yet understood. Since these fragments contain the Ras-association domain, it is possible that they act by associating with Ras. One possible theory is that Ras acts as a negative regulator of ASPP1 and ASPP2 by binding to them. Therefore deletion fragments that compete with full-length ASPP1/2 to bind to Ras could increase the activity of full-length ASPP proteins by preventing their binding to Ras. In this scenario, Ras would act as both a positive and negative regulator of the ASPP proteins: it would inhibit ASPP activity by binding to them and stimulate ASPP activity via the Raf-MAPK phosphorylation cascade. An integral part of this hypothesis is that the amino terminus fragments of ASPP1 and ASPP2 bind Ras. This could be shown by co-immunoprecipitating Ras and these deletion fragments. Another experiment that could confirm the competition binding theory is to see if increasing amounts of amino terminus fragments reduced full-length ASPP binding to Ras.

We have shown that full-length ASPP2 activity can be stimulated equally well by either human or mouse ASPP2 amino terminal fragments. As stated earlier, mouse and human ASPP2 are highly homologous and both contain the Ras-association domain in their first 90 amino acids. The amino terminus fragment of mouse ASPP2 used in the experiments shown in this chapter consists of residues 39-383. This fragment therefore does not contain an intact Ras-association domain, but is nonetheless able to stimulate full-length ASPP2 activity. This strongly suggests that the Ras-association domain of ASPP1 and ASPP2 is not involved in the stimulatory activity of the amino terminus fragments.

ASPP1 and ASPP2 both contain a putative alpha helix domain in their first 300 amino acids. Alpha helix domains are known to be involved in protein-protein interactions. It is therefore possible that ASPP1 and ASPP2 amino terminal fragments can relay their stimulatory activity to full-length ASPP not by competitively binding Ras via the Ras-association domain, but by binding some other protein via their alpha helical domain. These amino terminal fragments might even be able to form heterodimers with full-length ASPP1 and ASPP2. This can easily be tested with both *in vitro* and *in vivo* binding assays. It is not uncommon that binding of two proteins to each other can result in a change of the three-dimensional structure of one or both proteins. If the amino terminal fragments of ASPP1/2 bound full-length ASPP1/2, it would not be unlikely that the full-length ASPP proteins change their conformation as a consequence of the binding, therefore resulting in higher stimulatory effects. Since the putative alpha-helix domain is in the amino terminus of ASPP1 and ASPP2 and the p53-binding domain is in their carboxy-terminus, binding to the deletion fragments should not inhibit binding to p53 and might possibly enhance it by allosteric effects.

5.3.2. ASPP2 is a putative substrate to PKA kinase

Putative ASPP2 kinases were searched for by entering the protein sequence of ASPP2 on the web-site <http://scansite.mit.edu> that can recognize consensus phosphorylation sites. A small-scale screen was then performed by *in vitro* phosphorylation assays with a selected group of kinases. PKA was found to have a high probability of phosphorylating ASPP2 from the web-based search and *in vitro* phosphorylation confirmed that it phosphorylated a recombinant ASPP2 (693-1228) fragment with high activity. Using high performance liquid chromatography (HPLC) and mass spectrometry analysis, the residue of ASPP2 phosphorylated by PKA *in vitro* was found to be serine 737.

A construct was made of ASPP2 with the serine residues 736 and 737 mutated to alanine residues, preventing phosphorylation of ASPP2 on these sites *in vivo*. This phosphorylation mutant was found to have a similar activity to wild type ASPP2 in a transactivation assay. It is possible that ASPP2 phosphorylation mutant and wild type differ in their activity but their difference can only be seen under certain conditions. Assuming ASPP2 is activated by PKA by phosphorylation, a difference in the activity of wild type and phosphorylation mutant would only be seen when there is a high level of PKA stimulation. Thus forskolin was used to stimulate PKA.

Forskolin is a well-studied activator of adenylate cyclase. Its presence in cells leads to higher amounts of cAMP followed by increased PKA activity. When endogenous PKA is stimulated by forskolin, ASPP2 activity increases significantly and ASPP2 together with p53 transactivate the bax-luciferase reporter four-fold more effectively than in the absence of forskolin. Thus, PKA is an upstream positive regulator of ASPP2.

As PKA was shown to phosphorylate ASPP2 *in vitro* at serine 737 and PKA can stimulate ASPP2 *in vivo*, we tested whether this *in vivo* stimulation was via ASPP2 phosphorylation at serine 737. Unexpectedly, forskolin activated ASPP2 (S736,737A) phosphorylation mutant as efficiently as it did ASPP2 wild type. Therefore PKA-dependent stimulation of ASPP2 occurs independently of the phosphorylation status at serine 737.

Both *in vitro* phosphorylation data and the scanmotif web-site support the candidacy of PKA as a potential kinase for ASPP2. The medium-stringency search suggested that PKA phosphorylates ASPP2 with a high probability. Nonetheless, the list of putative phosphorylation sites on ASPP2 based on consensus sequences is not necessarily the best form of prediction. The medium stringency search showed MAPK1/Erk1 as a putative kinase for ASPP2 phosphorylation. This has proven to be correct, as shown in chapter 4. However, the search analysis also revealed that the site of ASPP2 to be phosphorylated by MAPK was serine 698. In fact, both *in vitro* and *in vivo* data presented in chapter 4 proved that MAPK phosphorylates ASPP2 on serine 827 only. Although these web-based searches for potential kinases are a good indication of kinases involved in the regulation of a particular protein, their suggestions should be taken cautiously.

Thus, the amino terminal fragments of ASPP and PKA have been found to be involved in ASPP regulation. Although I have shown in this chapter that they increase ASPP1 and ASPP2 activity markedly, the mechanism by which they regulate the ASPP proteins still requires some investigation.

Chapter 6

Final Summary and Discussion

6.1. ASPP1 and ASPP2 are novel Ras effectors

In its activated, GTP-bound form, Ras can recruit to the membrane and bind to an array of effector molecules, such as Raf, PI3K and RalGDS. As a consequence of their binding to Ras and their translocation to the plasma membrane, these effector proteins are activated and are then able to stimulate their own downstream effector molecules, leading to a variety of cellular responses. As well as these three well-characterized Ras effectors, many other proteins have been suggested to be putative Ras effectors. In most of these cases, however, their binding to Ras has been shown mainly in over-expression experiments or yeast two-hybrid binding assays and, to date, few results of functional significance have been discovered as a results of their binding Ras.

It is known that the amino terminal regions of ASPP1 and ASPP2 are necessary for their full activity (Samuels-Lev et al., 2001). By subjecting this amino-terminal region of the ASPP proteins to a BLAST search, I found that both ASPP1 and ASPP2 contain a putative Ras-association domain (RA) in their first 89 amino acids. This RA is also present in some of Ras effectors such as RalGDS, AF-6 and Nore1, and it mediates the interaction between these effectors and Ras (Hofer et al., 1994; Kikuchi et al., 1994; Kuriyama et al., 1996; Ponting and Benjamin, 1996; Spaargaren and Bischoff, 1994; Vavvas et al., 1998). This led me to hypothesise that ASPP1 and ASPP2 might interact with Ras. In accordance with this supposition, I have demonstrated that the amino terminus of ASPP1 can bind Ras directly, both *in vitro* and *in vivo*. The significance of

this interaction was confirmed when endogenous ASPP1 and ASPP2 were shown to bind endogenous Ras, after cells were stimulated with serum and growth factors.

For ASPP1 and ASPP2 to be Ras effectors, their activity must be stimulated after binding to Ras. Previous work in our laboratory has shown that ASPP1 and ASPP2 can co-activate p53 transactivation of apoptotic genes specifically (Samuels-Lev et al., 2001). I therefore used both luciferase transactivation assays and FACS analysis as means to measure Ras effect on the ASPP proteins. Tumour-derived, mutant H-RasV12 and K-RasV12 can both consistently increase ASPP1 and ASPP2 stimulation of p53 by 2-3 fold and inhibition of endogenous H-Ras or K-Ras by RNAi was sufficient to significantly reduce ASPP-mediated apoptosis in a p53-dependent manner. Therefore, not only can oncogenic Ras stimulate ASPP pro-apoptotic activity, but endogenous Ras is required for the full potential of ASPP-mediated apoptosis. It would be interesting to test whether oncogenic N-Ras can stimulate ASPP1 and ASPP2 to the same extent as its family members, H-Ras and K-Ras.

Thus, ASPP1 and ASPP2 have all the hallmarks of novel Ras effector proteins: they bind Ras directly through their RA domain, interact with Ras *in vivo* and their activity is stimulated by active Ras.

6.2. ASPP1 and ASPP2 are novel MAPK substrates

Since Ras is upstream of a number of different pathways, I enquired whether ASPP1 and ASPP2 activation by Ras might be mediated by one of these downstream pathways. Indeed, I discovered that activated Raf could stimulate ASPP activity to the same extent as oncogenic Ras, strongly suggesting that Ras activation of ASPP is mediated by the Ras-Raf pathway. In accordance with ASPP being regulated by the Ras-Raf pathway, both ASPP1 and ASPP2 contain a conserved MAPK phosphorylation site. I have shown that MAPK phosphorylates ASPP1 and ASPP2 *in vitro* and *in vivo*, on serines 746 and 827, respectively, and that phosphorylation of these serines by MAPK results in increased ASPP activity.

MAPK is known to have several substrates, both in the cytoplasm and in the nucleus. MAPK phosphorylation of the transcription factors TCF and Ets2 in the nucleus results in an increase of their transactivation activity (Gille et al., 1992). On the other hand, phosphorylation of the kinase Rsk by MAPK in the cytoplasm, results in Rsk translocation to the nucleus where it can then phosphorylate and activate the transcription factor CREB (Xing et al., 1996). Thus, MAPK phosphorylation of its substrates can increase their transcriptional activity and induce their translocation from the cytoplasm to the nucleus.

In the case of ASPP1 and ASPP2, however, MAPK was shown to increase their activity by other means: MAPK-mediated ASPP phosphorylation led to an increase in ASPP2 protein levels. This suggests that MAPK can stimulate its substrates by increasing their stability, and consequently, their activity. Nonetheless, it is still possible that MAPK phosphorylation of ASPP1 and ASPP2 might have more than a single effect and that,

following phosphorylation, the ASPP proteins can translocate to the nucleus. So far, ASPP1 and ASPP2 have only rarely been shown to be present in the nucleus by cell staining, although their presence on the promoters of p53 pro-apoptotic target genes has been observed by chromatin immunoprecipitation (ChIP) analysis (Samuels-Lev et al., 2001). Activating MAPK by oncogenic Ras transfection or by endogenous Ras stimulation has not revealed any change of ASPP1 and ASPP2 localization into the nucleus. However, it is possible that the conditions tested could be improved and the effect of MAPK on ASPP localization should be tested using protease inhibitors, nuclear export inhibitors and/or caspase inhibitors. Since it is common for protein-protein interaction to be regulated by phosphorylation, it is also possible that MAPK phosphorylation of ASPP1/2 could potentially affect their ability to bind p53. This could be tested by comparing the effectiveness of co-immunoprecipitating p53 with either wild-type or phosphorylation mutant ASPP following Ras stimulation. One would expect that in a situation where the ASPP proteins bind p53 with higher affinity, they would have an increased ability to co-activate p53 pro-apoptotic function. This could provide another possible explanation for ASPP increased activity following MAPK phosphorylation.

We know that MAPK phosphorylation of ASPP1 and ASPP2 leads to an increase in their protein levels. However, this needs not be an exclusive mechanism for MAPK to increase ASPP activity; it is possible that MAPK can regulate ASPP at many different levels, as is the case with Mdm2 regulation of p53.

6.3. Linking the two pathways – is there a feedback loop?

So far, I have shown that ASPP1 and ASPP2 are direct effectors of Ras and that they are also downstream targets of the Raf-MAPK pathway. This poses a paradox – how can ASPP be immediately downstream of Ras and simultaneously downstream of MAPK? One possible explanation for this apparent inconsistency is that ASPP is involved in a feedback loop. One such model would be that, following phosphorylation by MAPK, ASPP would then bind Ras and either stimulate its activity or inhibit its activity, depending on whether it is a positive or negative feedback.

To try to investigate this issue, *ASPP2*-null mouse embryo fibroblasts (MEFs) were used to test whether ASPP had any effect in Ras stimulation of MAPK. Figure 4.16 showed that Ras was able to stimulate MAPK phosphorylation with similar kinetics in the presence or absence of ASPP2. However, interestingly, the basal level of MAPK phosphorylation was higher in *ASPP2* knockout cells. One possible explanation might be that ASPP2 could be involved in a negative feedback loop with the Raf-MAPK pathway. Thus, following Ras-Raf-MAPK activation, ASPP2 would be transiently stimulated and would prevent its further stimulation by inhibiting MAPK activity. In this circumstance, ASPP2 would not get stimulated sufficiently to co-activate p53. However, in cases where Ras is constitutively activated, ASPP2 would have no effect on diminishing the MAPK signal and it would therefore be fully activated to induce p53-dependent apoptosis (figure 6.1). It is not uncommon that some Ras effectors are only partially stimulated by normal Ras and fully stimulated by oncogenic Ras, as seen with PI3K (McCormick, 1999; van Weering et al., 1998). In this scenario, it would be expected that phosphorylated ASPP2 has a higher affinity for Ras than non-

phosphorylated ASPP2, since it would only act in a negative feedback loop as a result of its own activation.

Nonetheless, the idea that ASPP2 might act in a negative feedback loop with Ras following its phosphorylation by MAPK is speculative. Both ASPP2 wild-type and phosphorylation mutant can co-localize with Ras at the plasma membrane. An immunoprecipitation would be critical to determine whether Ras associates preferentially to the phosphorylated or non-phosphorylated form of ASPP2, therefore resolving the issue of whether ASPP needs to be activated by MAPK prior to binding Ras.

There are many Ras mutants that can specifically bind to some of its effectors and not others (White et al., 1995). It would be worthwhile to screen these mutants for their binding to ASPP1 and ASPP2. If a Ras mutant that can no longer bind to ASPP1/2 but can still activate the Raf-MAPK pathway can be identified, it would be very useful to test whether ASPP is involved in a feedback loop with the Raf-MAPK pathway; if ASPP can negatively regulate the MAPK pathway then the mutant Ras would stimulate MAPK more efficiently than normal, activated Ras. Conversely, if ASPP positively regulates the MAPK pathway, then the mutant Ras would be less effective in stimulating MAPK.


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graph TD
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    Ras --> Down[ ]
    style Down fill:none,stroke:none
  
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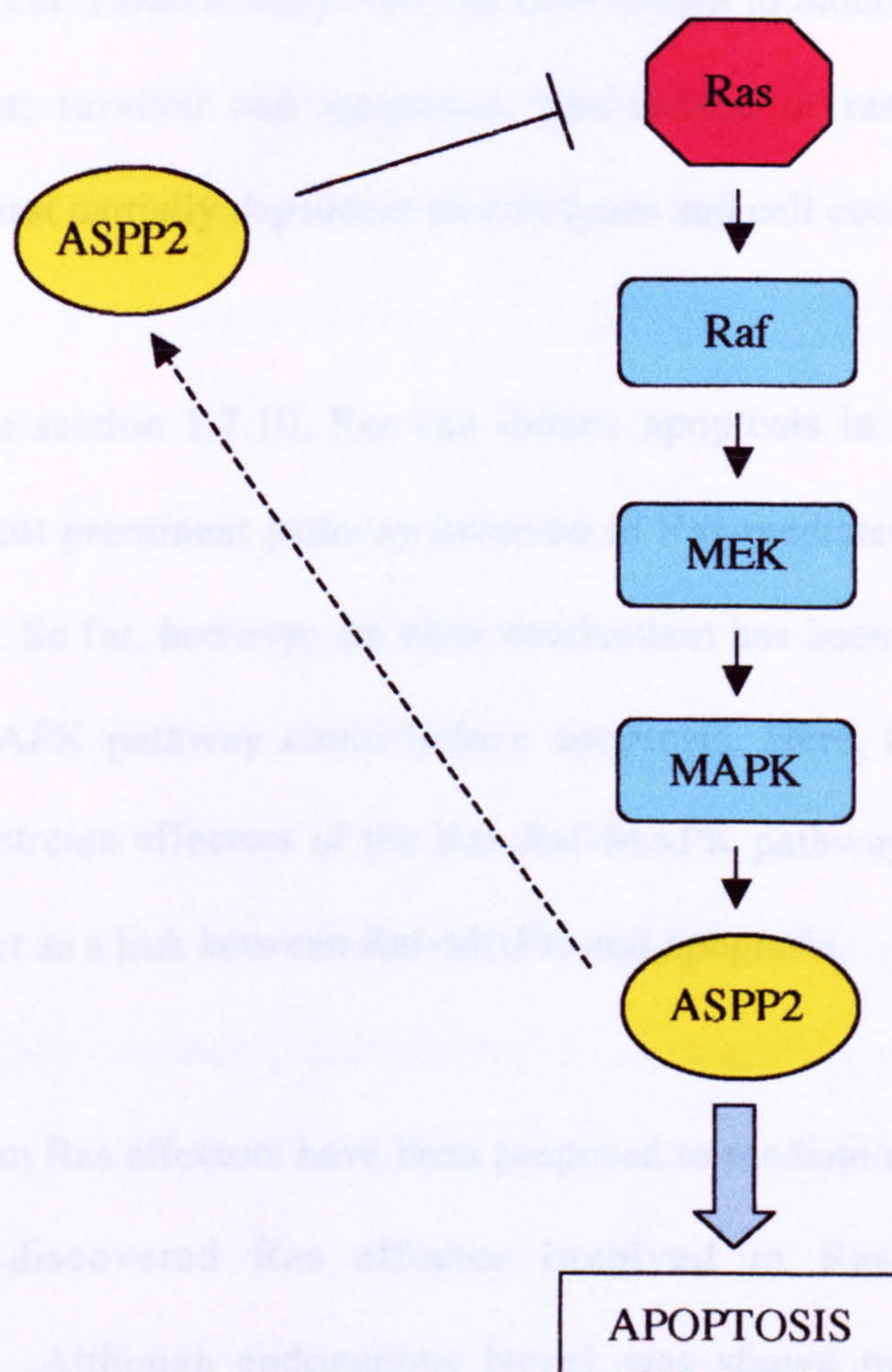


Figure 6.1. *Model for Ras-ASPP2 negative feedback.* The Ras-Raf-MAPK pathway stimulates ASPP2 activity by phosphorylation. Activated ASPP2 can then bind to Ras and prevent its further activation of the Raf-MAPK pathway, thereby acting in a negative feedback loop.

6.4. A novel pathway for Ras-mediated apoptosis

As a result of Ras binding and activation of its effectors, a number of different responses can occur. Paradoxically, Ras has been shown to induce cell proliferation and cell cycle arrest; survival and apoptosis. The choice of response following Ras activation is at least partially dependent on cell types and cell context.

As mentioned in section 1.7.10, Ras can induce apoptosis in a number of different contexts. The most prominent pathway involved in Ras-mediated apoptosis is the Raf-MAPK pathway. So far, however, no clear mechanism has been established to explain how the Raf-MAPK pathway could induce apoptosis. Here, I identify ASPP1 and ASPP2 as downstream effectors of the Ras-Raf-MAPK pathway and propose that the ASPP proteins act as a link between Raf-MAPK and apoptosis.

Other downstream Ras effectors have been proposed to mediate apoptosis. Nore1 is the most recently discovered Ras effector involved in Ras-mediated apoptosis (Khokhlatchev). Although endogenous Nore1 was shown to be in complex with endogenous Ras after cells were stimulated with serum, the rest of the study was done with overexpression systems. The authors clearly demonstrated that oncogenic K-RasV12 and a mutant form of oncogenic H-RasV12 (H-rasGV12, E37G) could stimulate apoptosis. However, they were unable to provide a mechanism related to the Nore1/Mst1 pathway. Similarly, the MEKK-JNK pathway is also known to induce apoptosis following Ras stimulation, but the mechanism involved is still unclear. Thus, ASPP1 and ASPP2 provide the first clear mechanism of Ras-mediated apoptosis.

The PI3K downstream effector of Ras has been shown to be involved in survival signalling. It is known that the survival signals of the PI3K-PKB pathway could counterbalance the apoptotic signals of the Raf-MAPK pathway. It would be interesting to investigate whether the PI3K pathway could inhibit the Raf-MAPK-ASPP1/2 apoptotic pathway. Preliminary data using an activated form of the p110 catalytic subunit of PI3K were inconclusive: p110 alone could stimulate p53 activity significantly in transactivation assays, and therefore its effect on ASPP co-activation of p53 was not observed. Additionally, Saos2 cells transiently transfected with p53 and ASPP2 and treated with the PI3K inhibitor LY294002 showed inconsistent effects using a luciferase reporter system (data not shown).

Ras is known to stimulate p53 activity (see section 1.8). Through the induction of Arf and PML, Ras most commonly stimulates p53-mediated cell cycle arrest in primary cells, not apoptosis (Ferbeyre et al., 2000; Kamijo et al., 1997; Pomerantz et al., 1998). However, it is not uncommon for oncogenes to induce p53-mediated apoptosis: E1A, E2F1 and myc can all three induce apoptosis in a p53-dependent manner (Debbas and White, 1993; Hermeking and Eick, 1994; Qin et al., 1994; Wagner et al., 1994; Wu and Levine, 1994). In this thesis, I present a novel pathway in which Ras stimulates p53, not to induce cell cycle arrest, but to stimulate apoptosis. The ability of oncogenes to stimulate cell cycle arrest or apoptosis allows the cell to have a fail-proof system. Thus, following activation of an oncogene, instead of inducing the cell to proliferate in an uncontrolled manner, oncogenes would activate tumour suppressor proteins, which would then prevent the cell from replicating its damage. Although cell cycle arrest and apoptosis are both efficient manners by which cells can prevent propagation of their

damage, apoptosis is irreversible and therefore the more effective of the two mechanisms.

Hence, the presence of a mutant Ras would stimulate ASPP1 and ASPP2 via the Raf-MAPK pathway. The activated ASPP proteins would then be in a position to co-activate p53 to induce apoptosis (figure 6.2B). In cells where p53 or the ASPP proteins are inactivated by mutation or by reduced expression, oncogenic Ras would then be able to induce uncontrolled growth (figure 6.2C). One would therefore expect that a cell harbouring an activating mutation of Ras would select for an inhibitory mutation of either p53 or ASPP. Indeed, in colorectal cancer, K-Ras mutation occurs predominantly before p53 mutation (Kinzler and Vogelstein, 1996). In accordance with this model, *p53*-null fibroblasts are highly susceptible to Ras transformation (Hicks et al., 1991). It would be interesting to test the levels of ASPP1 and ASPP2 in tumours that contain a mutant Ras and wild-type p53; if this model is correct, we would expect to find either inactivating mutations of ASPP or reduced expression of these proteins in these tumours. So far, ASPP has rarely been found to be genetically altered (Mori et al., 2000) but its expression is reduced in many human breast carcinomas containing p53 wild-type (Samuels-Lev et al., 2001).

To further analyze the role ASPP plays in Ras transformation, *ASPP2*-null MEFs can be used. Preliminary studies have shown that there is a difference in colony formation following infection of oncogenic Ras in MEFs with wild-type ASPP2 compared to *ASPP2*-null MEFs (data not shown). This is in accordance with the model that ASPP2 plays a role in inhibiting Ras transformation.

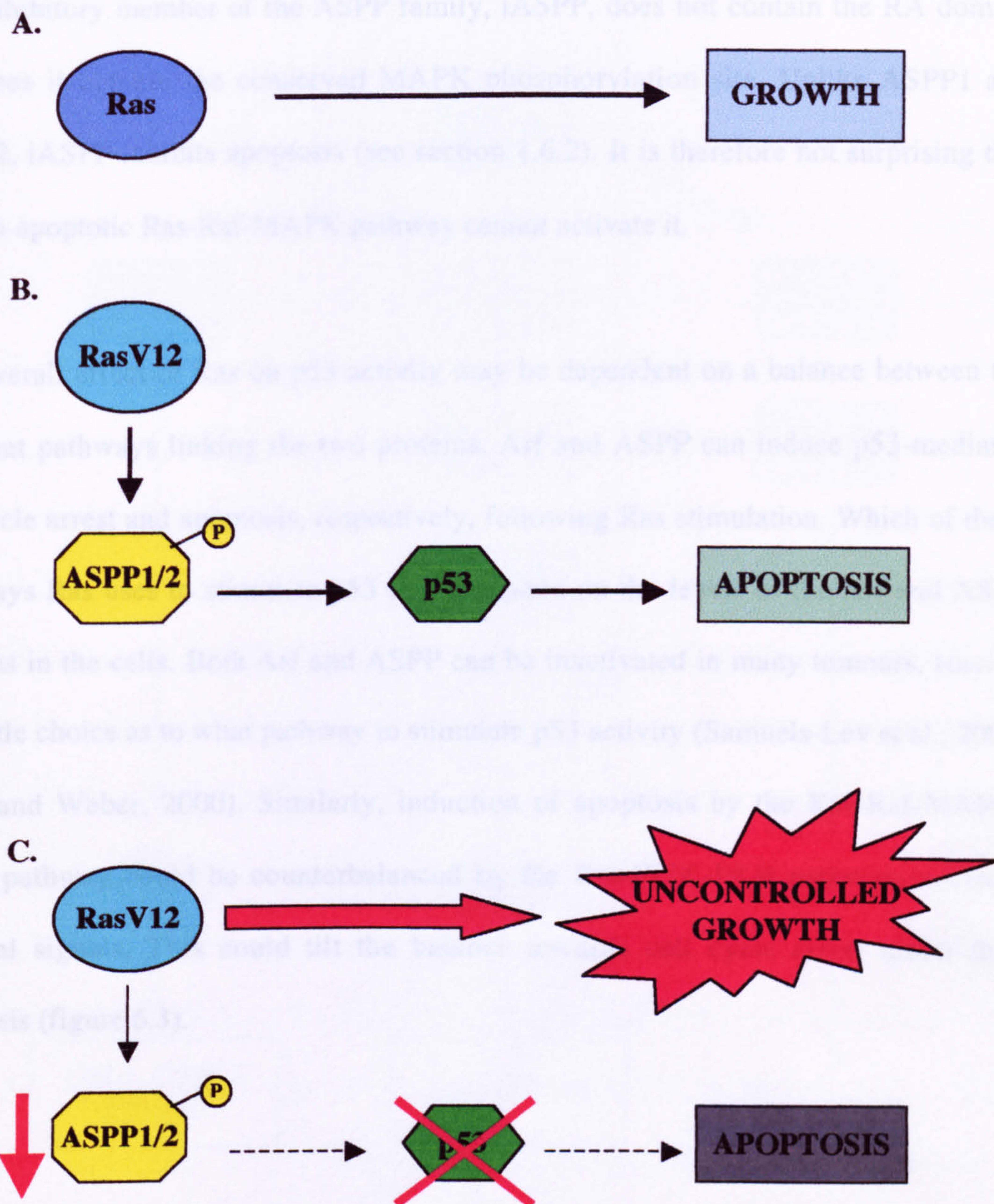


Figure 6.2. *Oncogenic Ras induces apoptosis in an ASPP- and p53-dependent manner.* (A) Normal Ras stimulates normal growth and does not stimulate ASPP1/2. (B) Oncogenic Ras stimulates ASPP1 and ASPP2 to co-activate p53 and induce apoptosis. (C) In cells with either reduced levels of ASPP expression or p53 mutations, oncogenic Ras is no longer able to induce apoptosis in an ASPP- and p53-dependent manner and will therefore stimulate uncontrolled growth.

The inhibitory member of the ASPP family, iASPP, does not contain the RA domain nor does it contain the conserved MAPK phosphorylation site. Unlike ASPP1 and ASPP2, iASPP inhibits apoptosis (see section 1.6.2). It is therefore not surprising that the pro-apoptotic Ras-Raf-MAPK pathway cannot activate it.

The overall effect of Ras on p53 activity may be dependent on a balance between the different pathways linking the two proteins. Arf and ASPP can induce p53-mediated cell cycle arrest and apoptosis, respectively, following Ras stimulation. Which of these pathways Ras uses to stimulate p53 might depend on the levels of the Arf and ASPP proteins in the cells. Both Arf and ASPP can be inactivated in many tumours, leaving Ras little choice as to what pathway to stimulate p53 activity (Samuels-Lev et al., 2001; Sherr and Weber, 2000). Similarly, induction of apoptosis by the Ras-Raf-MAPK-ASPP pathway could be counterbalanced by the Ras-PI3K-PKB pathway-mediated survival signals. This could tilt the balance towards cell cycle arrest rather than apoptosis (figure 6.3).

6.4. Therapy

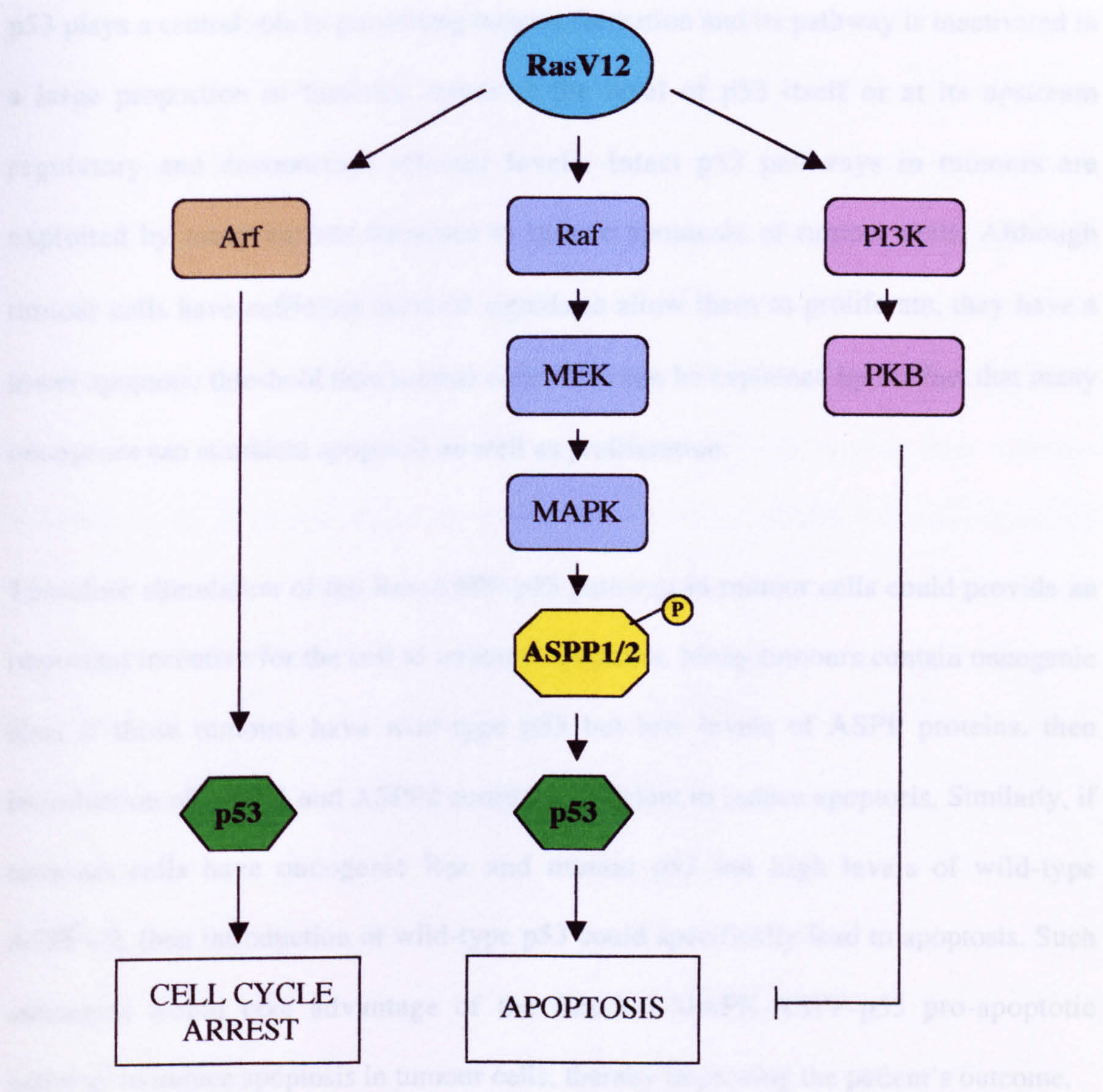


Figure 6.3. *Ras stimulates p53 to induce cell cycle arrest and apoptosis.* Ras can stimulate Arf to induce p53-dependent cell cycle arrest. It can also stimulate ASPP1 and ASPP2 to induce p53-dependent apoptosis. Through its PI3K/PKB pathway, Ras can inhibit p53-mediated apoptosis. The end response of Ras stimulation depends on the balance of all these different pathways.

6.4. Therapy

p53 plays a central role in preventing tumour formation and its pathway is inactivated in a large proportion of tumours, either at the level of p53 itself or at its upstream regulatory and downstream effector levels. Intact p53 pathways in tumours are exploited by many current therapies to induce apoptosis of tumour cells. Although tumour cells have sufficient survival signals to allow them to proliferate, they have a lower apoptotic threshold than normal cells. This can be explained by the fact that many oncogenes can stimulate apoptosis as well as proliferation.

Therefore stimulation of the Ras-ASPP-p53 pathway in tumour cells could provide an important incentive for the cell to undergo apoptosis. Many tumours contain oncogenic Ras; if those tumours have wild-type p53 but low levels of ASPP proteins, then introduction of ASPP1 and ASPP2 could be sufficient to induce apoptosis. Similarly, if tumours cells have oncogenic Ras and mutant p53 but high levels of wild-type ASPP1/2, then introduction of wild-type p53 could specifically lead to apoptosis. Such strategies would take advantage of the Ras-Raf-MAPK-ASPP-p53 pro-apoptotic pathway to induce apoptosis in tumour cells, thereby improving the patient's outcome.

As Albert Camus said: "There is only one serious philosophical problem. It is suicide. To judge whether life is or is not worth living". In the case of tumour cells, it is beneficial for the whole organism if they choose to die.

6.5. Summary of achievements in this study

In this thesis I have described how ASPP1 and ASPP2 are regulated by Ras. I have shown that ASPP1 and ASPP2 are novel Ras effectors: they bind directly to Ras via their amino terminal region, both *in vitro* and *in vivo*, and their pro-apoptotic activity is stimulated by oncogenic Ras. Additionally, endogenous Ras is necessary for full ASPP1/2-dependent apoptosis and cisplatin-induced apoptosis. I have also shown that ASPP1 and ASPP2 are activated by the Raf-MEK-MAPK downstream effector pathway of Ras, and that MAPK directly phosphorylates the ASPP proteins at their carboxy-terminal region. Phosphorylation of ASPP1 and ASPP2 by MAPK results in increased protein levels and, consequently, an increase in their activity. I have also briefly investigated other mechanisms of ASPP regulation, namely PKA and ASPP deletion fragments.

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